



## Development of Industrial Yeast for Second Generation Bioethanol Production

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# Development of Industrial Yeast for Second Generation Bioethanol Production

Risø-PhD-Report

Xiaoru Hou  
Risø-PhD-92(EN)  
July 2011

Risø DTU  
National Laboratory for Sustainable Energy

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# **PREFACE**

I was officially transferred to the present Ph.D. project from January 1, 2009. The work presented in this Ph.D. thesis was conducted from January 1, 2009 to July 31, 2011 in the Microbial Engineering (MIC) Research Group, Biosystems Division, Risø National Laboratory for Sustainable Energy, DTU.

This dissertation consists of (1) a short summary; (2) a chapter of general introduction describing the theoretical background of the project and reviewing the related literatures and results of the Ph.D. project (Chapter 1); (3) four chapters presenting the results obtained in the Ph.D. project (Chapter 2 – Chapter 5). Results based on Chapter 2 and Chapter 4 have been published in Applied Microbiology and Biotechnology.

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Xiaoru Hou

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November 2011

## SUMMARY

The cost of lignocellulose-based bioethanol needs to be reduced, in order to commercialize this clean and sustainable fuel substitute for fossil fuels. A microorganism that can completely and efficiently convert all the sugars in lignocellulose into ethanol is one of the prerequisites of a cost-effective production process. In addition, the microorganisms should also have a high tolerance towards the inhibitory compounds present in the lignocellulosic hydrolysate, which are formed during the pretreatment of lignocellulose. Baker's yeast, *Saccharomyces cerevisiae*, is generally regarded as a robust microorganism and can efficiently ferment glucose. But it lacks the ability to ferment xylose which comprises 20-35% of lignocellulose. Naturally xylose-fermenting yeast such as *Pichia stipitis* is much more sensitive to inhibitors than *S. cerevisiae* and it requires accurately controlled microaerophilic conditions during the xylose fermentation, rendering the process technically difficult and expensive.

In this study, a novel xylose fermenting yeast *Spathaspora passalidarum* displayed fast cell growth and efficient xylose fermentation under anaerobic conditions. In contrast, *P. stipitis* was almost unable to utilize xylose under the same conditions. It is further demonstrated that *S. passalidarum* converts xylose by means of NADH-preferred xylose reductase (XR) and NAD<sup>+</sup>-dependent xylitol dehydrogenase (XDH). Thus, the capacity of *S. passalidarum* to utilize xylose under anaerobic conditions is possibly due to a balance between supply and demand of cofactor through this XR-XDH pathway. Only one other XR with NADH preference has been reported so far. Unfortunately, *S. passalidarum* also has a low tolerance towards inhibitors generated during pretreatment, which prevents immediate use of this yeast in industrial application. *S. passalidarum* is able to convert the inhibitor furfural to furfuryl alcohol in a synthetic medium when the addition of furfural is low. The enzymes involved in furfural and 5-hydroxymethylfurfural (HMF) reductions by this yeast have both cofactor preferences for NADH. Due to the low inhibitor tolerance, the

growth of *S. passalidarum* was completely inhibited in the liquid fraction of pretreated corn stover and wheat straw.

The inhibitor tolerance of *S. passalidarum* was improved by the method of genome shuffling including UV mutagenesis and protoplast fusion. The protoplast of a UV-induced furfural-resistant mutant of *S. passalidarum* (*S. passalidarum* M7) was fused with the protoplast of a robust yeast *S. cerevisiae* ATCC 96581. The finally selected hybrid strain (FS22) has desired phenotypes derived from both parents, namely the ability to ferment xylose from *S. passalidarum* and an increased tolerance to inhibitors from *S. cerevisiae* ATCC 96581. Phenotypic and molecular analysis indicated that *S. passalidarum* M7 was the dominant parental contributor to the hybrid. Rearrangement of DNA segments from the other parental strain *S. cerevisiae* ATCC 96581 possibly occurred in FS22.

The inhibitor tolerance of the robust yeast *S. cerevisiae* ATCC 96581 was further improved by sequentially adapting this strain into media with increasing amounts of the liquid fraction of pretreated corn stover (CSLQ). The adapted strain completely fermented glucose in 100% CSLQ and the ethanol yield was 0.48 g/g glucose, while the parental strain was unable to ferment under this condition. Co-fermentation of this adapted strain with the selected protoplast fused hybrids (FS2 or FS22) in the pretreated wheat straw hydrolysate improved the final ethanol yield by 11% and 26%, respectively, due to partial conversion of xylose in the hydrolysate by the xylose-fermenting hybrids. Co-fermentation with one robust C6 fermenting yeast for detoxification and one C5 fermenting yeast for converting xylose into ethanol could be a viable strategy for lignocellulosic bioethanol production.

## RESUMÉ

Omkostningerne ved produktion af lignocellulose-baseret bioethanol skal nedbringes, før dette rene og bæredygtige brændstof kan markedsføres som erstatning for fossile brændstoffer. Mikroorganismer, der fuldstændigt og effektivt kan omdanne alle sukkerstoffer i lignocellulose til ethanol, er en af forudsætningerne for billig produktion. Derudover bør mikroorganismen også have en høj tolerance over for de inhiberende stoffer i det lignocellulose hydrolysat, som bliver dannet under forbehandlingen af lignocellulose. Bagegær, *Saccharomyces cerevisiae*, betragtes generelt som en robust mikroorganisme, der effektivt kan fermentere glukose, men denne gær mangler evnen til at fermentere xylose, som udgør 20-35 % af lignocellulose. Naturligt xylose-forgærende gær som *Pichia stipitis* er langt mere følsom over for inhibitorer end *S. cerevisiae*. For at bruge *Pichia stipitis* kræves nøje kontrollerede mikroaerofile forhold under xylose-gæringen, hvilket gør processen teknisk vanskelig og dyr.

I denne undersøgelse blev det påvist, at en ny xyloseforgærende gær, *Spathaspora passalidarum*, under anaerobe forhold har god cellevækst og effektivt forgærer xylose. I modsætning hertil var *Pichia stipitis* kun dårligt i stand til at udnytte xylose under de samme betingelser. Det påvistes endvidere, at *S. passalidarum* omdanner xylose ved hjælp af en xylose reductase (XR) med præference for NADH og en NAD<sup>+</sup>-afhængig xylitol dehydrogenase (XDH). Denne evne til at forgære xylose under anaerobe forhold skyldes muligvis cofaktor balancen i den XR-XDH katalyserede reaktionsvej. Kun én anden XR med NADH præference er indtil videre kendt. Desværre har *S. passalidarum* også en lav tolerance overfor inhibitorer, der dannes under forbehandlingen af lignocellulose. Den lave tolerance forhindrer umiddelbart brugen af denne gær i industriel skala. *S. passalidarum* er i stand til at konvertere inhibatoren furfural til furfurylalkohol i et syntetisk medium, når mængden af furfural er lav. De enzymer i denne gær, der er involveret i furfural og 5-hydroxymethylfurfural (HMF) reduktioner, har begge præference for at bruge NADH

som co-faktor. På grund af den lave inhibitor tolerance er væksten af *S. passalidarum* fuldstændigt hæmmet i den flydende fraktion af forbehandlet majs- og hvedehalm.

Inhibitortolerancen af *S. passalidarum* blev forbedret ved ”genome-shuffling”, der involverer UV-mutagenese og protoplastfusion. Protoplaster af en UV-induceret furfural-resistent mutant af *S. passalidarum* (*S. passalidarum* M7) blev fusioneret med protoplaster fra en robust gær *S. cerevisiae* ATCC 96581. Den endeligt selekterede hybridstamme (FS22) har den ønskede fænotype, der stammer fra begge forældre, nemlig evnen til at fermentere xylose fra *S. passalidarum* og en øget tolerance over for inhibitorer fra *S. cerevisiae* ATCC 96581. Fænotypisk og molekylær analyse viste, at *S. passalidarum* M7 var den dominerende bidrager til hybrid. Re-arrangement af DNA-segmenter fra den anden forældre-stamme, *S. cerevisiae* ATCC 96581, har muligvis også fundet sted i FS22.

Inhibitortolerancen af den robuste gær *S. cerevisiae* ATCC 96581 blev yderligere forbedret ved gradvist at tilpasse stammen til vækstmedier indeholdende stigende mængder af den flydende fraktion af forbehandlet majs- og hvedehalm (CSLQ). Den tilpassede stamme forgærede glukose fuldstændigt i 100% CSLQ, og ethanoludbyttet var 0,48 g/g glukose. Udgangsstammen var ikke i stand til at forgære glukose under disse betingelser. Co-fermentering i det forbehandlede hvedehalm-hydrolysat med den tilpassede stamme og med protoplast hybriderne (FS2 eller FS22) forbedrede det endelige ethanoludbytte med henholdsvis 11% og 26%, sandsynligvis på grund af delvis forgæring af xylose i hydrolysatet, udført af de xylose forgærende hybrider. Co-fermentering med en robust C6-forgærende gær og med en C5-forgærende gær, der kan omdanne xylose til ethanol, kunne være en bæredygtig strategi for at gøre lignocellulose baseret bioethanol produktion mere effektiv.



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# Chapter 1

## Introduction

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### 1.1 What is bioethanol and why is bioethanol?

Bioethanol is an ethanol derived exclusively from fermentation of sugar components in plant material, considered to be renewable.

Bioethanol has been suggested as a fuel, especially for transportation use, for more than 100 years. In 1860 the first internal combustion engine fueled by ethanol was invented by Nicholas August Otto. During 1880s, cars fueled by bioethanol produced from corn starch were designed by Henry Ford (Kovarík 1998). From the beginning of the 20<sup>th</sup> century until around 1940s bioethanol was blended with gasoline and used as transport fuel in the USA, Brazil and many European countries (e.g. Germany, France, and England) (Kovarík 2006). At that time, engineers working on engine compression and knock issues were especially interested in ethanol. Henry Ford, founder of the Ford Motor Company, and Charles Kettering, inventor of the electrical starting motor and leaded gasoline, believed bioethanol would be the “fuel of the future” (Kovarík 2006; Kovarík 1998). However, after World War II, fewer and fewer countries in the world continued blending ethanol with gasoline, due to the discovery of abundant reserves of crude oil. The low price of fossil fuels made the production and use of ethanol as a fuel uneconomical. Until 1973, when the first oil crisis occurred, Brazil was the only country in the world staying with the ethanol blending program (Kovarík 2006).

After the occurrence of the two oil crisis, the first in 1973 and the second in 1979, people began to realize the importance of alternative energy sources. Today, the growing concerns of the long-term fossil fuel availability (Greene et al. 2002), the rising concern over global warming problems and the increasing attention to the national energy security (Cox and Hug 2010) have lead to the widespread opinion that clean and sustainable energy substitutes for fossil fuels are required. Bioethanol is thus back in focus as a fuel substitute. The benefits of bioethanol are generalized as the following:

### **(1) Promotion of agriculture**

Bioethanol which is produced either from crops or from crop residues has the potential to promote the development of agriculture and agro-industry and to create new job opportunities in rural areas. It has been estimated that around 6 to 10 jobs are created for every thousand tons of bioethanol produced (Sasson 2005). However, the bioethanol production is often linked to agricultural policy.

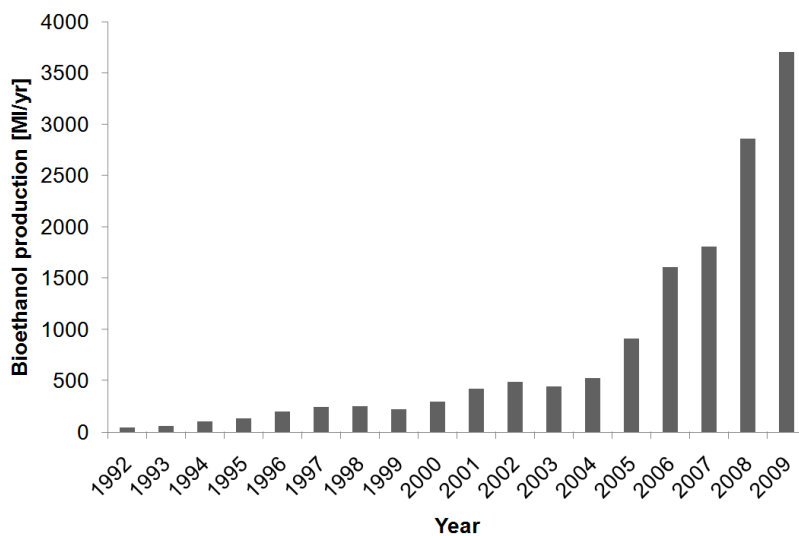
### **(2) Alleviation of dependence on fossile fuels**

Nowadays, it becomes more and more urgent for western countries to eliminate the heavy dependence on fossil fuel import. Therefore, the development of alternative energy sources is of significant importance, involving critical political issues that are beyond the scope of this dissertation.

### **(3) Reduction of air pollution**

Bioethanol is a much cleaner fuel than fossil fuels, as the combustion of the latter generates air pollutants such as sulfuric dioxide ( $\text{SO}_2$ ), nitric oxide (NO) and volatile organic hydrocarbons (VOCs) which affect the environment in destructive ways. Nitric oxide can be oxidized into nitrogen dioxide in the atmosphere. VOCs form peroxy radicals and react with NO to produce nitrogen dioxide ( $\text{NO}_2$ ). In sunlight,  $\text{NO}_2$  dissociates into O and NO. The O reacts with  $\text{O}_2$  to

produce ozone. This cycling of  $\text{NO}_2$  and  $\text{NO}$  means that even small concentrations of nitrogen oxides ( $\text{NO}$  and  $\text{NO}_2$ ) can produce large amounts of ozone when VOCs are present. Ozone together with methane, water vapor, and carbon dioxide are the primary greenhouse gas (GHG) in the atmosphere of earth which can cause global warming problems. In addition,  $\text{NO}_2$  and  $\text{SO}_2$  are the major precursors of acid rain which acidifies soil and water, and accelerates corrosion of buildings. High concentration of sulfuric dioxide can also result in breathing problems.



**Figure 1.1** Bioethanol productions in the EU-27 from 1992 to 2009 (Source: <http://www.plateforme-biocarburants.ch/en/infos/eu-bioethanol.php>, accessed 4 July 2011)

Bioethanol production in the European Union (EU) has increased dramatically from 2004 (Figure 1.1). The year before, the EU had issued Directive 2003/30/EC on promoting the use of biofuels or other renewable fuels for transport as a mean to reduce the GHG emission from the transportation sector. In 2010 the EU set a target of reducing 60% to 80% of GHG emission by 2050. In order to reach this ambition and for the consideration of national security, the Danish government announced that Denmark is to be totally fossil fuel independent in 2050 (page 5, Energy strategy 2050: from coal, oil and gas to green energy 2011). In Denmark, a total of 80% of the energy consumption came from fossil fuels in 2008. This implies that substitutes for fossil fuels are very important to guarantee Denmark to fulfill its zero-fossil fuel target. Among the possible

energy substitutes, biofuels including bioethanol derived from biomass, can play an important role, not only in the transport sector, but also as a backup for the fluctuating production from wind turbines (Danish commission on climate change policy 2010).

Traditionally, bioethanol is produced from crop sugars such as sugar cane juice and corn starch. This technology is termed as 1<sup>st</sup> generation bioethanol. Many industrial bioethanol plants based on this technology have been operated all around the world e.g. USA, Brazil, Germany, Sweden and China. However, the major road-block for further use of first generation bioethanol is that this use of crops competes with food production for limited agricultural land. This has led to a heated and lasting debate, mainly on moral issues. Moreover, a further increase in the production of the crop-based bioethanol will lead to higher prices of the crops and this will increase the price of the bioethanol significantly. Thus, it is important that the dependency on crops is reduced (Waltz 2007), underlining the importance of development of a second generation technology, which put efforts on using presumptively cheaper and more price-stable biomass residues, e.g. grass, wood chips, and crop residues for bioethanol production. Furthermore, it is estimated that the biomass residue accounts for about 50% of the plant biomass in the world (Claassen et al. 1999). The annual production of the plant biomass is estimated to be  $1 \times 10^{10}$  MT worldwide (Sanchez et al. 2008), which is a much larger pool of feedstock than crops for the bioethanol production. However, based on today's technology, the cost of 2<sup>nd</sup> generation bioethanol is high compared with the cost of fossil fuels. Extensive developments for reducing the process cost of 2<sup>nd</sup> generation bioethanol are required. It is worth to mention here that although bioethanol is more expensive than fossil fuels, the real cost of the latter does not integrate the costs of the conflicts generated by oil exploitation, the costs of shore and sea contamination by oil and oil-tanker wreckage, and the costs of the air pollution caused by combustion of fossil fuels.

## 1.2 Current process for second generation bioethanol production

Second generation bioethanol is produced from fermentation of sugars in lignocellulosic biomass residues.

### 1.2.1 Lignocellulose composition

The word “lignocellulose” describes the three main components of the plant biomass: cellulose, hemicellulose and lignin. Ash and small amount of proteins, oils and waxes take up a minor amount in the composition of plant biomass. The composition and structure of lignocellulose is affected by the type and the age of the plants.

Cellulose is the most abundant natural polymer on earth and it constitutes 35-50% of lignocellulose. It is a linear polymer, composed of  $\beta$ -D-glucose linked by  $\beta$ -(1, 4)-glucosidic bonds. Cellulose is always associated with a variety of other polysaccharides and other polymers, such as starch, pectin, lignin and a variety of hemicellulose. This architecture protects cellulose from hydrolysis (Zaldivar et al. 2001).

Hemicellulose constitutes 20-35% of lignocellulose. It is composed of a linear or branched backbone of sugar monomers, predominantly pentoses, e.g. D-xylose and L-arabinose, and small amounts of hexoses, e.g. D-mannose and D-galactose, depending on the plant species (Zaldivar et al. 2001). As a rule of thumb, xylose is the main hemicellulose sugar in herbaceous biomass such as wheat straw and corn stover (Lee et al. 2007).

Lignin takes up 10-25% of lignocellulose and is the most complex compound. It is a three dimensional, highly branched aromatic polymer with the substituents connected by both ether and carbon-carbon linkages. It is composed of three principal building blocks: *p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol), and sinapyl alcohol (syringyl propanol). In herbaceous biomass, lignin is mainly composed of guaiacyl-, syringyl-, and *p*-hydroxyphenyl-units (Jeffries 1990).



The exact content of each lignocellulosic component varies between different lignocelluloses. However, for economic reasons, the ideal kind of lignocellulose for bioethanol production should contain high amounts of sugar polymers, i.e. cellulose and hemicellulose.

### **1.2.2 Process of the second generation bioethanol production**

Based on today's technology, the production of bioethanol from lignocellulose requires at least three steps: pretreatment to break down the lignocellulosic structure, enzymatic hydrolysis to release free sugars from cellulose and hemicellulose, and fermentation to convert the sugars to ethanol. After that, the produced ethanol can be recovered by distillation and purified to meet different fuel specifications.

#### **1.2.2.1 Pretreatment**

Lignocellulose provides a vast source of fermentable sugars for bioethanol production. At present, releasing fermentable sugars by enzymatic hydrolysis is recognized as the most attractive method, with only minor environmental effects. However, the complex structure of lignocellulose, which protects the living plants against physical attacks (e.g. rain and wind) or microbial degradation, makes the contact of enzymes with cellulose and hemicellulose difficult. Therefore, before enzymatic hydrolysis can be applied, such structural and compositional barriers have to be removed or altered to separate the cellulose and hemicellulose parts. This step is termed as "pretreatment" (Mosier et al. 2005). In general, cellulose is much more difficult to be made accessible to enzymes than hemicellulose, due to the tight wrapper of protective hemicellulose-lignin components outside cellulose.

There are multitudes of different pretreatment methods because of the diversity of the different physical-chemical characteristics in different lignocelluloses. In principle, these methods can be classified into three groups: physical pretreatment, chemical pretreatment and biological

pretreatment. There can also be methods combining above ways such as physical-chemical pretreatment.

Physical pretreatment mainly includes mechanical comminution and pyrolysis. The goal of the mechanical comminution is to enhance the efficiency of enzymatic hydrolysis by reducing the crystallinity and reducing the particle size of the biomass to increase the contact area between cellulose and enzymes. Different milling processes, i.e. dry milling, wet milling, balling milling and vibro energy milling can be used (Taherzadeh and Karimi 2008; Sun and Cheng 2002). However, the energy requirement of milling is relatively high and this process does not seem to be economically feasible (Hendriks and Zeeman 2009). Pyrolysis includes different methods such as hydrothermal treatment and extrusion. During the hydrothermal treatment, the material is heated with high-pressure steam permeating the lignocellulose and initiate auto-hydrolysis reactions (Thomsen et al. 2008). In this study, the lignocellulosic substrate, i.e. corn stover and wheat straw, were pretreated by a hydrothermal treatment in Inbicon A/S (DONG Energy subsidiary, Denmark). Extrusion is a well established process in food industries and it has recently been used as a physical pretreatment method for lignocellulosic biomass. During the extrusion, the biomass is subjected to heating, mixing and shearing, resulting in physical and chemical modifications, which increase the accessibility of carbohydrates to enzymatic attack (Karunanithy et al. 2008).

Chemical pretreatment includes many kinds of chemical solvents that can improve the enzymatic accessibility of cellulose by removing hemicelluloses-lignin barriers. The most commonly used reagents are acids, e.g.  $\text{H}_2\text{SO}_4$  and  $\text{HCl}$ , alkalis, e.g.  $\text{NaOH}$  and  $\text{KOH}$ , and oxidants, e.g.  $\text{H}_2\text{O}_2$  and  $\text{O}_3$ . Acid pretreatment can efficiently solubilize the hemicellulose fraction and make the cellulose more accessible to enzymes. Alkali pretreatment increases the cellulose digestibility due to an increase of the internal surface by swelling, reduction of the polymerization degree, and destruction of the links between lignin and other polymers (Galbe and Zacchi 2007). Pretreatment with

powerful oxidants showed high delignification efficiency, which increases the sugar yield in the following enzymatic hydrolysis. However, the requirement for large amounts of oxidants makes the process less economical (Sun and Cheng 2002)

Biological pretreatment mainly refers to degradation by microorganisms such as brown-rot and white-rot fungi. Brown-rot fungi (*e.g. Coniophora prasinoides* and *Leucogyrophana arizonica*) mainly attack cellulose (Highley 1980), while white-rot fungi (*e.g. Phanerochaete chrysosporium*, *Trametes versicolor*, *Ceriporiopsis subvermispora*, and *Pleurotus ostreatus*) attack both cellulose and lignin (Taniguchi et al. 2005). White rots are the most effective and extensively studied fungi for biological pretreatment. The advantages of biological pretreatment are the low energy requirement and the low negative environmental effects. However, the rates of degradation in most biological pretreatment processes are too low and an increase in the degradation rate is required for this kind of pretreatment to be applied at industrial level (Sun and Cheng 2002).

Physical-chemical pretreatment combines both physical and chemical processes. Normally these methods are considered as more effective than a physical process or a chemical process alone. Examples of such pretreatment methods are SO<sub>2</sub> steam explosion, steam explosion with addition of peroxide, CO<sub>2</sub> explosion and ammonia fiber explosion (AFEX) (Alvira et al. 2010).

In the current pilot-scale lignocellulosic bioethanol production, the physical-chemical pretreatment methods are the most frequently adopted. However, generation of high amounts of inhibitory compounds and corrosion problems of the equipment are drawbacks of these methods (Sun and Cheng 2002). Inhibitory compounds are generated as a result of the degradation of released sugars and lignin components, due to the harsh conditions (high temperature, high pressure, addition of oxidants and acids/alkalis), and these inhibitory compounds can reduce the efficiency of enzymatic hydrolysis (Kim et al. 2011) and seriously inhibit the microbial fermentation and ethanol production (Palmqvist and Hahn-Hägerdal 2000b).

Generally speaking, the pretreatment generated inhibitory compounds can be divided into 3 categories: (1) furans such as furfural and hydroxymethyl furfural (HMF), (2) weak acids such as acetic acid and formic acid, and (3) phenolic compounds such as aromatic and polyaromatic compounds with various substituents. More specifically, furfural is formed by dehydration of pentoses released from hemicellulose, while HMF is formed by dehydration of hexoses released from cellulose and hemicellulose. Acetic acid is formed by de-acetylation of hemicelluloses, while formic acid is formed by HMF or furfural breakdown. Varieties of phenolic compounds are formed due to the lignin breakdown or carbohydrate degradation (Almeida et al. 2007).

The types and amounts of inhibitory compounds present in pretreated lignocellulosic hydrolysate vary according to the composition of the lignocellulose and the pretreatment conditions such as temperature, time, pressure, pH, redox conditions and addition of catalysts (Mosier et al. 2005). For example, lignocellulose pretreated under dilute acid conditions will generate significantly more HMF than under alkali or neutral conditions (Almeida et al. 2007). The inhibitor composition of corn stover and wheat straw hydrolysate pretreated under the identical conditions are different (Chapter 5). Generally speaking, a wider range of different phenolic compounds are formed after pretreatment as compared with furans and weak acids, but the concentrations of phenolics are far lower than furans and weak acids (Palmqvist and Hahn-Hägerdal 2000b). Mechanisms of the inhibition of these compounds have been studied for decades, but only few have been clarified, as reviewed in Section 1.3.2.2.1.

#### **1.2.2.1.1 Detoxification**

Due to the deleterious effects of the inhibitory compounds to the whole bioethanol production process, a detoxification step following the pretreatment is often added to remove these chemicals. The detoxification method can be physical, chemical or biological (Palmqvist and Hahn-Hägerdal

2000a). Physical detoxification methods include evaporation and extraction. For example, ether extraction efficiently removed acetic acid, furfural, HMF and many phenolic compounds such as vanillin from steam-pretreated aspen wood and improved the ethanol yield from 0 to 93% of that obtained in a reference fermentation (Wilson et al. 1989). Phenol extraction of pretreated woods improved the efficiency of enzymatic hydrolysis to its full potential (Kim et al. 2011). Chemical detoxification method is mainly alkali treatment. It is in general conducted by increasing the pH of the pretreated hydrolysate to 9-10 by addition of alkali such as NaOH (Nilvebrant et al. 2003), KOH or  $\text{Ca(OH)}_2$  (overliming) (van Zyl et al. 1988) and readjusting pH to around 5 by addition of  $\text{H}_2\text{SO}_4$ . The ethanol yield and productivity are improved after this kind of treatment due to the precipitation of toxic compounds (Nilvebrant et al. 2003). Biological detoxification methods comprise application of enzymes and microbial detoxification (Parawira and Tekere 2010). Application of peroxidase and laccase can help to remove phenolic compounds (Larsson et al. 1999b) and increase the ethanol yield and productivity (Chandel et al. 2007). A variety of microorganisms have the ability to degrade toxic compounds in the pretreated hydrolysate. As an example, the thermophilic bacterium *Ureibacillus thermosphaericus* was able to degrade furfural, HMF and phenolic compounds present in a waste house wood hydrolysate (Okuda et al. 2008). A fungal strain *Amorphotheca resinae* ZN1 degraded all kinds of toxins on various pretreated lignocellulose feedstocks such as corn stover, wheat straw, rice straw, cotton stalk and rape straw (Zhang et al. 2010). In addition, baker's yeast *Saccharomyces cerevisiae* also has the ability to metabolise some toxic compounds (e.g. furfural) to the corresponding less toxic compounds (e.g. furfuryl alcohol) (Palmqvist and Hahn-Hägerdal 2000b).

Although the addition of a detoxification step improved the final ethanol yield and productivity, the prolonged processing time and the additional cost of agents, operation and equipment maintenance make the whole process less economical. Therefore, microorganisms that can tolerate

high concentration of inhibitors (i.e. can *in situ* detoxify high concentration of inhibitors) as well as efficiently produce ethanol is required (see more details in Section 1.3.2.2). The entire process can thereafter be simplified and the extra cost by the additional detoxification step can be saved.

#### **1.2.2.2 Enzymatic hydrolysis**

After pretreatment, hydrolysis is required to release free sugars from cellulose and hemicellulose. Hydrolysis can be done either by weak acid hydrolysis or by enzymes. Weak acid hydrolysis uses dilute acids ( $\text{H}_2\text{SO}_4$  and  $\text{HCl}$ ) at low concentration (normally between 0.5~1.5%) and high temperature (normally 200~240°C) (Sanchez and Cardona 2008), requiring lots of energy and producing high amounts of inhibitors degraded from sugars (Pienkos and Zhang 2009). Enzymatic hydrolysis is considered to be a more promising method because inhibitors are not generated and reaction conditions are mild.

Hemicellulose is much easier hydrolysed than cellulose and therefore, the conversion of cellulose into glucose by cellulases is the most important procedure during the enzymatic hydrolysis. Cellulases are usually a mixture of several enzymes, including at least three major groups: endoglucanases, exocellobiohydrolases and  $\beta$ -D-glucosidases (Sun and Cheng 2002). The hydrolysis of cellulose is the result of the synergistic action of these three groups of enzymes: First, the endoglucanases (EGs) break the cellulose chains randomly to shorten the cellulose chains and make new ends available for the exocellobiohydrolases (CBHs); second, the exocellobiohydrolases cut down cellobiose units from the ends of the cellulose chains; third,  $\beta$ -glucosidases (BGs) release the glucose monomers from cellobiose (Sun and Cheng 2002). Accumulation of cellobiose can cause end-product inhibition on CBHs. Therefore addition of BGs is necessary to solve this problem. An optimal ratio of EGs, CBHs and BGs can enhance the sugar yield and rate of hydrolysis as well as reduce the costs of enzyme addition (Mansfield et al. 1999).

Fungi such as *Sclerotium rolfii*, *Phanerochaete chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* are used for cellulases production, among which *Trichoderma sp.* are considered to be the most productive (Balat 2011). Other fermenting microorganisms such as some species of bacteria *Clostridium*, *Cellulomonas* and *Bacillus* also produce cellulases (Sun and Cheng 2002), providing a future possibility of converting cellulose into ethanol without addition of extra enzymes (see details in Section 1.3.1).

Up to now, the mechanism of total lignocellulosic saccharification has not been completely clear, although extensive research has been conducted on the enzymatic hydrolysis process concerning the kinetics, catalytic actions, and interactions between enzymes and their substrates (Balat 2011; Sun and Cheng 2002). The heterogeneous nature of the structures of lignocellulosic matrices makes it difficult to fully understand the interactions between enzyme complexes and these substrates (Sun and Cheng 2002; Mansfield et al. 1999). However, many factors have been identified that affect the efficiency of enzymatic hydrolysis. The most important ones are substrates, enzyme activities, and reaction conditions. Starting with low substrate levels, an increase of the substrate concentration leads to an increase of sugar yield and reaction rate, but substrate inhibition occurs when the substrate concentration increases to high levels (Sun and Cheng 2002). The extent of substrate inhibition depends on the nature of the cellulosic substrate as well as that of the enzyme (Huang and Penner 1991). Moreover, different physical and chemical characteristics of the substrate such as accessible surface area, cellulose fiber crystallinity, and lignin and hemicellulose content decide the susceptibility of cellulosic substrates to cellulases (Mansfield et al. 1999). Increasing the enzyme activity by means of increasing the dosage of enzyme can improve the yield and rate of hydrolysis, but this also adds to the costs of the process. Therefore, an optimal dosage of enzyme is required to obtain as high sugar yield as possible at a reasonable enzyme cost (Gregg and Saddler 1996). Cellulase loading of 10~30 filter paper unit (FPU) per gram cellulose are most frequently

applied in laboratory studies (Georgieva et al. 2008; Chen et al. 2007), while industrial requirements for enzyme loading should be as low as possible. The addition of surfactants such as Tween (Wu and Ju 1998) or polyethylene glycol (PEG) (Sipos et al. 2010) has been found to help reducing the dosage requirement of cellulases. The effect is caused by surfactants by modifying the cellulose surface and minimizing the irreversible bindings of cellulases to cellulose. Cellulase works on cellulose in three steps: (1) binding of the cellulases to cellulose; (2) degrading the cellulose; and (3) releasing the binding cellulases. Some binding of cellulases to cellulose is irreversible, resulting in the loss of cellulase during hydrolysis and increasing the demand of enzyme dosage (Converse et al. 1988). Finally, different cellulases have different optimal pH and temperature. Conducting enzymatic hydrolysis at their optimal pH and temperature has positive effects on yield and rate of the hydrolysis. For most commercial available cellulases the optimal pH generally lies between pH 4 and 5 and the optimal temperature lies around 50°C (e.g. Novozymes Celluclast 1.5L and Genencor Accellerase 1500).

### **1.2.2.3 Fermentation**

After hydrolysis, the liberated sugars (i.e. hexoses and pentoses) are converted to ethanol by microbial fermentations. Baker's yeast, *S. cerevisiae*, is a preferred ethanol producer due to the low level of by-products and its high ethanol tolerance. Yeast fermentation of crop sugars (1<sup>st</sup> generation) is a mature technology. However, these traditional yeast fermentations are not suitable for the fermentation requirements of 2<sup>nd</sup> generation lignocellulosic bioethanol production. The basic problems are: (1) the hydrolysate contains many growth inhibiting compounds from the pretreatment process and (2) there is a large fraction of pentoses (i.e. mainly xylose) that can not be fermented by *S. cerevisiae*. In order to solve these problems, improvements of both the fermenting microorganisms and fermentation technology are required.



A vast variety of microorganisms (bacteria, yeast, and fungi) are able to ferment xylose into ethanol. For example, anaerobic bacteria have the capacity to ferment a broad range of sugars such as glucose, xylose and cellobiose (Strobel et al. 1995; Cook et al. 1994). However, these bacteria have vital disadvantages preventing them being used in the lignocellulosic ethanol production. These disadvantages include: (1) a mixed acid product formation where ethanol is a minor product; (2) being severely inhibited by pretreatment-generated inhibitors, sugars (at concentration around 5%) and ethanol (at concentrations exceeding 2%); (3) susceptibility to infections due to the requirement of neutral pH (i.e. around 6~7) for growth (Hahn-Hägerdal et al. 1994). Some species of filamentous fungi are capable of fermenting hexoses and pentoses into ethanol (Wu et al. 1986). However, the poor ethanol tolerance and extremely low sugar conversion rate are drawbacks for these strains in industrial applications (Hahn-Hägerdal et al. 2007). Xylose fermenting yeasts are regarded as the most promising microorganism to be utilized in fermenting xylose part in the lignocellulosic hydrolysate, due to their high ethanol tolerance, high osmotic tolerance, resistance to infections because of the requirement of acidic pH (around 5) for growth, and most importantly, the similar growth requirements as *S. cerevisiae*. This final point renders it easier to integrate these yeasts into the present bioethanol production facilities developed for *S. cerevisiae*. However, although traditional xylose fermenting yeast species as *Pichia* and *Candida* have been studied for decades, none of them have been applied in the industry up to date. One of the major reasons is the requirement for precisely controlled oxygenation by these yeasts during the xylose fermentation (see details in Section 1.3.2.1.1), and this is technically difficult under large-scale industrial conditions. Metabolic engineering has been applied to improve the phenotypes of different production microorganisms, e.g. deleting the byproducts formation pathways in anaerobic bacteria (Taylor et al. 2009), or introducing xylose fermentation pathways into *S. cerevisiae* (see details in Section 1.3.2.1.4). However, all the recombinant strains are still far from the industrial requirements

and none have been employed in industrial scale production (Nevoigt 2008). Therefore, the lack of a suitable fermenting microorganism that can efficiently convert all the sugars present in lignocellulosic hydrolysate into ethanol is still one of the bottlenecks for developing the technology of 2<sup>nd</sup> generation bioethanol. Section 1.3 describes additional details about the physiology and the progress in the yeast strain improvement.

Two fermentation approaches are traditionally applied in bioethanol production: one is called separate hydrolysis and fermentation (SHF) and the other is called simultaneous saccharification and fermentation (SSF) (Galbe and Zacchi 2002). In the SHF process, the enzymatic hydrolysis of cellulose and hemicellulose is performed at the optimal conditions for the enzyme complex, and the fermentation is subsequently conducted at the optimal conditions for the fermenting microorganisms. In the SSF process, the enzymatic hydrolysis and fermentation are carried out together at suboptimal conditions for both hydrolysis and fermentation. Both SHF and SSF have advantages and the disadvantages (Table 1.1), and therefore the choice of approach relies on many factors such as the nature of the lignocellulosic biomass, the equipment and the properties of enzymes and the fermenting microorganisms. For the purpose of lowering production capital cost, another fermentation approach termed as “consolidated bioprocess (CBP)” has been suggested, where the cellulase production, cellulose hydrolysis and fermentation of all sugars are combined in one step. Section 1.3.1 tells more details about the concept of this approach.

Regarding the industrial operations, there are also three main modes for conducting fermentation: batch, fed-batch, and continuous fermentation. In the batch mode, fermentation is conducted in a single bioreactor from start to finish. It is a prevailing operation applied in industry due to the advantage of low equipment cost, low control requirement and easiness of handling (Sanchez and Cardona 2007). In the fed-batch mode, the substrate is fed at intervals such that the volume of substrate varies during the fermentation. This mode can prevent substrate inhibition by

maintaining a low substrate concentration (Parawira and Tekere 2010). In the continuous mode, substrate (influent) is pumped continuously into the reactor where the fermenting microorganism is working. The product (effluent) is taken out continuously and recovered. It can lead to high ethanol productivity and a long period of effective production. However, the risks of contamination as well as accumulating mutations in microorganisms are high due to the long cultivation periods (Jedrzejewska and Kozak 2011).

**Table 1.1** Comparison of SHF and SSF <sup>a</sup>

Approaches	Pros	Cons
Separate hydrolysis and fermentation (SHF)	<ol style="list-style-type: none"> <li>1. Carrying out each step under optimal conditions</li> <li>2. Providing possibility of running continuous fermentation for a long time with cell recycling</li> <li>3. Liquefying the solid substrates in the enzymatic hydrolysis step so that reducing the problems of mass and heat transfer in the fermentation step</li> </ol>	<ol style="list-style-type: none"> <li>1. Reducing the sugar yield from enzymatic hydrolysis due to the end-product inhibition of cellulases caused by glucose accumulation</li> <li>2. Increasing the complexity of total process</li> </ol>
Simultaneous saccharification and fermentation (SSF)	<ol style="list-style-type: none"> <li>1. Reducing the end-product inhibition of cellulases caused by glucose accumulation</li> <li>2. Reducing the total capital cost</li> <li>3. Decreasing the contamination risks</li> </ol>	<ol style="list-style-type: none"> <li>1. Carrying out both enzymatic hydrolysis and fermentation under the suboptimal conditions</li> <li>2. Causing difficulty in recycling and reusing the microorganisms since they are mixed with the fiber residues</li> </ol>

<sup>a</sup>. Summarized from Rudolf et al. 2005 and Galbe and Zacchi 2002

### 1.3 Economic prospect of the process

Bioethanol production based on lignocellulose has not been demonstrated on commercial scale so far, mainly due to the high production costs as compared with fossil fuels. The economic prospects of lignocellulosic bioethanol have been investigated and big variations have been found in

different studies (Shafiei et al. 2011; Sassner et al. 2008; Wingren et al. 2003; Aden et al. 2002). The variations can be due to the fluctuations in the capital and operational costs of different processes and also in the costs of feedstock. Although the price of feedstock from biomass residues is considered to be low, the availability of feedstock is significant for the estimation of the production costs and this estimation tends to be geographically constrained (Lavigne and Powers 2007). In some studies, costs of ethanol production are estimated below 0.6 euro per litre ethanol (Shafiei et al. 2011; Sassner et al. 2008; Wingren et al. 2003). In some other studies, the cost of using commercial enzymes is around 0.4 euro per litre ethanol produced (Bryant 2010). Therefore, the figures for ethanol production costs below 0.6 euro/litre may be underestimated. As an example, the cost of lignocellulosic ethanol production in a demonstration plant of Inbicon (DONG energy subsidiary, Kalundborg, Denmark) may be estimated. 50 million euro has been invested in this plant and it has about 30 employees. It has the capacity of handling 30000 tons/year of wheat straw, annually producing 5.4 million litres of ethanol (Henrikson 2010). The capital costs plus manpower may be estimated at around 6 million euro/year, resulting in the cost of around 1 euro per litre ethanol produced. This estimation does not include items difficult to estimate, e.g. the value of 13100 tons of lignin pellets and 11250 tons of molasses also produced and not the costs for raw materials (wheat straw), enzymes, water, energy and transportation. However, it does indicate that big cost reductions are required for commercialization of second generation bioethanol. The strategies and the progress to minimize the process cost are reviewed in the following section.

### **1.3.1 Process improvement**

As described above, the present bioethanol production process comprises pretreatment (and often detoxification), enzymatic hydrolysis and fermentation steps. All the steps require improvements to minimize the production costs.

A cost effective pretreatment should include the following characteristics (Alvira et al. 2010; Mosier et al. 2005):

maximizing the recovery of pentose (hemicellulose) fractions

minimizing sugar decomposition or loss

minimizing the formation of inhibitor compounds to the enzymes and fermenting microorganisms

minimizing the energy demand

minimizing the capital investment on equipment maintenance

minimizing the by-production of solid-waste residues and waste water

In order to meet the targets listed above, efforts have been made to combine different physical-chemical pretreatments, such as room temperature pretreatment with O<sub>3</sub> generated plasma assistance (Schultz-Jensen et al. 2011) or steam explosion pretreatment with addition of acetic acids (Xu et al. 2010). Five of the most frequently employed pretreatment processes (dilute acid, hot water, ammonia fiber explosion, ammonia recycle percolation and lime) are described and compared by Eggeman and Elander (2005). However, so far all the designs for pretreatment are capital intensive, as reduced costs in some aspects (e.g. sugar recovery or equipment investment) often are counterbalanced by higher costs in other aspects (e.g. energy demand, inhibitor formation, or catalyst recovery).

The enzymatic hydrolysis is the least understood step in the whole production process of lignocellulosic bioethanol (Merino and Cherry 2007) and the cost of this step is also high. For example, it is estimated that the cost of converting 80% of cellulose in acid pretreated corn stover by commercial cellulases is around 0.4 euro per litre ethanol produced until 2010 (Bryant 2010). During recent years, the efforts have focused on improving the efficiency of known enzymes, identifying new enzymes, combining enzyme complexes at optimal ratios to certain lignocellulosic

substrates and minimizing enzyme production costs (Garcia-Aparicio et al. 2007; Merino and Cherry 2007).

In a fermentation step performed with traditional baker's yeast, the presence of inhibitors and the significant amounts of unfermented pentose (mainly xylose) are highlighted as two main problems affecting the process costs. Current corn-to-ethanol processing (1<sup>st</sup> generation) plants typically produce ethanol at 10% to 14% w/w. These ethanol levels are at least two to three fold higher than those produced from lignocellulose using current technology, due to the limits on glucose concentrations in lignocellulosic biomass. In order to make the final titre of lignocellulosic ethanol comparable to that of corn-to-ethanol processing, one possible solution could be increasing the dry matter (DM) of the lignocellulose slurry. As an example, Inbicon's industrial demonstration (status September 2010) stated that it is capable of pretreating wheat straw of 35% dry matter (Henriksen 2010), which can yield around 15% glucose, resulting in around 7% to 8% ethanol if all glucose can be released and completely fermented. However, high dry matter content often gives rise to levels of inhibitory compounds which create difficulties during enzymatic hydrolysis and fermentation. In addition, the rheological properties of a very dense fibrous suspension may cause problems for mass and heat transfer (Rudolf et al. 2005), and require higher investments in equipment. Fermenting the significant amount of xylose part in biomass can help to reduce the concentration of dry matter of lignocellulose. Take wheat straw as an example, since there is around 40% of glucose and 20% of xylose in biomass, only 20% to 25% dry matter of wheat straw is required to achieve 7% to 8% ethanol production. Significant efforts have been made to improve the desired properties, i.e. inhibitor tolerance and capacity of utilizing pentose, of fermenting microorganisms. Compared with bacteria and other fungi, yeast was recognized as the most promising production host (Section 1.2.2.3; Rumbold 2009; Delgenes et al. 1996). The progresses of strain improvement of yeast are reviewed in details in Section 1.3.2.

A concept termed “consolidated bioprocessing (CBP)” was proposed by Lynd et al. (1996) and has attracted more and more attentions. In this process, cellulase production, cellulose hydrolysis and fermentation of C5 and C6 sugars are combined in a single step. The design of this process is based on the assumption that eliminating separate process steps may reduce capital and operating costs and may allow other synergistic benefits (Lynd et al. 2005). However, the realization of this concept strongly depends on either using an ideal microorganism that is capable of efficiently converting cellulose into ethanol, or using simultaneously a microbe degrading lignocellulose to sugars and a microbe fermenting sugars to ethanol. Although some bacteria (e.g. *Clostridium thermocellum*) (Fang and Ouyang 2010) and fungi (e.g. *Aspergillus terreus*) (Pushalkar and Rao 1998) have the capacity to convert cellulose into ethanol and some recombinant yeast strains secreting cellulases have been constructed (Apiwatanapiwat et al. 2011; Yanase et al. 2010), the extremely low cellulose conversion rates and extremely low product yields are still challenges to make CBP a success. Therefore thorough understanding of genetics and physiology and subsequent manipulation of numerous cellular traits to construct an ideal microorganism are demanded.

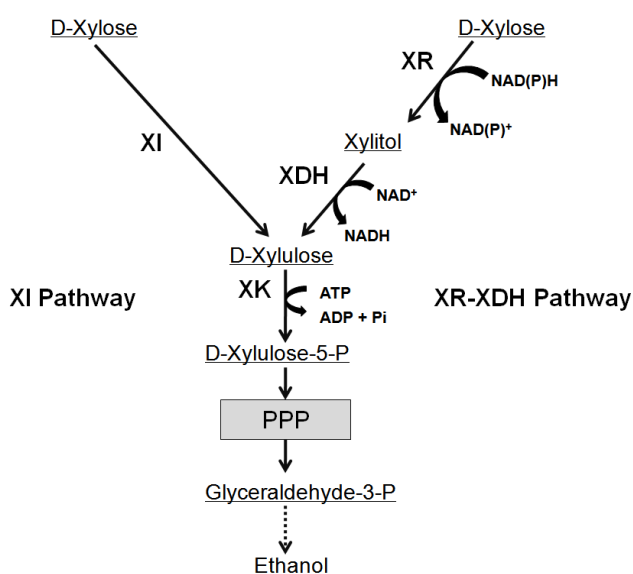
### **1.3.2 Strain improvement**

A suitable fermenting microorganism plays a key role in reducing the cost not only in the fermentation step, but may also lead to a design of a novel production process (e.g. CBP process). In this section, the progress in improvement of yeast strains to overcome two problems, i.e. unfermented xylose and microbial inhibition by inhibitors present in the pretreated lignocellulosic hydrolysate, is reviewed.

#### **1.3.2.1 Xylose fermentation**

##### **1.3.2.1.1 Xylose fermentation pathway**

Some species of bacteria, yeast and fungi can naturally ferment xylose to ethanol. Figure 1.2 schematically illustrates the metabolic pathway of xylose fermentation in bacteria, yeast and fungi. Xylose is converted to xylulose which is then phosphorylated into xylulose-5-phosphate (X5P) by catalysis of xylulokinase. X5P enters into pentosophosphate pathway (PPP) and is further metabolized into ethanol. In most xylose fermenting fungi (e.g. *Pichia stipitis* and *Candida shehatae*) xylose is converted to xylulose by two oxidoreductases, xylose reductase (XR) and xylitol dehydrogenase (XDH), involving cofactors NAD(P)H and NAD<sup>+</sup>, respectively (XR-XDH pathway, Figure 1.2) (Hahn-Hägerdal et al. 2007). That is, xylose is first reduced to xylitol by NAD(P)H-dependent XR, and then xylitol is oxidized to xylulose by NAD<sup>+</sup>-dependent XDH. In bacteria, on the other hand, xylose is metabolized into xylulose through another pathway. Xylose is directly isomerised into xylulose by xylose isomerase (XI), with no cofactor requirement (XI pathway, Figure 1.2). A few fungi such as *Piromyces sp.* strain E2 (Harhangi et al. 2003) have been reported to harbour XI activity. A XI was also purified from a yeast strain *Candida boidinii* that can grow on methanol (Vongsuvanlert and Tani 1988).

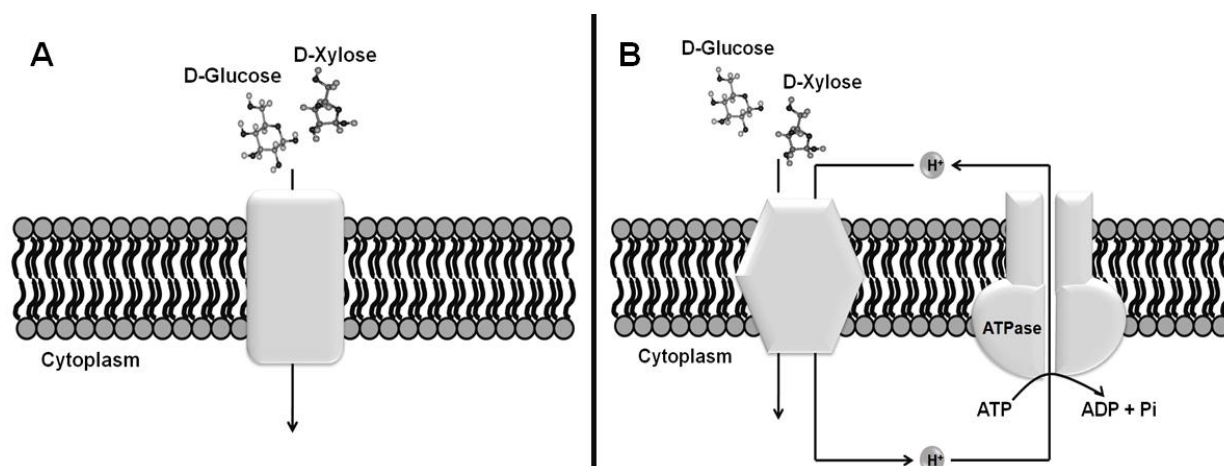


**Figure 1.2** Xylose fermentation pathways in bacteria, yeast and other fungi. XI: xylose isomerase; XK: xylulokinase; XR: xylose reductase; XDH: xylitol dehydrogenase. XI pathway is mainly adopted by bacteria and XR-XDH pathway is mainly adopted by fungi. Figures are modified from Hahn-Hägerdal et al. (2007).



### 1.3.2.1.2 Xylose transport

Xylose uptake by yeasts requires transport proteins. Transportation can be either facilitated diffusion in which a sugar gradient is the only driving force (Figure 1.3A), or a proton symport (Figure 1.3B) (Jojima et al. 2010). In traditional baker's yeast *Saccharomyces cerevisiae*, xylose is poorly taken up due to the xylose being transported by nonspecific hexose transporters through facilitated diffusions. The affinity of the hexose transporters for xylose is one to two orders of magnitude lower than that for glucose (Kötter and Ciriacy 1993). Therefore, xylose uptake usually occurs after depletion of hexose sugars in the medium, as is the case for recombinant xylose fermenting *S. cerevisiae* strains (Matsushika et al. 2009; Karhumaa et al. 2007; Gárdonyi et al. 2003b). In naturally xylose-fermenting yeasts, xylose can also be taken up through active transport using high-affinity xylose transporters (Jojima et al. 2010). In some xylose-fermenting yeasts, glucose can be transported as well through this active transport system (Gárdonyi et al. 2003a). However, this transport system concomitantly consumes 1 mol of ATP for each proton cotransported with xylose (Figure 1.3B). Therefore, under anaerobic conditions, the depletion of conserved ATP may happen and reduce the anaerobic growth (Weusthuis et al. 1993). In naturally xylose-fermenting yeasts, it is common that different transport systems are used under different conditions. For example, *Kluyveromyces marxianus* displays the activity of both active transport and facilitated diffusion of xylose under aerobic conditions while only facilitated diffusion of xylose occurs under microaerophilic conditions (SpencerMartíns 1994). In *Candida succiphila* a single high-affinity transporter is active in a medium with only xylose while a low-affinity transporter is active in a medium with glucose (SpencerMartíns 1994). *Spathaspora passalidarum* (Nguyen et al. 2006) also shows different xylose transport systems under anaerobic and aerobic conditions. This was suggested by the simultaneous utilization of glucose and xylose under aerobic conditions and glucose-preferred utilization under anaerobic conditions (Chapter 2).



**Figure 1.3** General view of xylose transportation system in yeast. (A) facilitated transport; (B) proton symport transport. Figures are modified from Hahn-Hägerdal et al. (2007).

### 1.3.2.1.3 Naturally xylose fermenting yeast

Large numbers of yeast species have capacity to metabolize xylose. But only approximately 1% of them are able to ferment xylose into ethanol (Hahn-Hägerdal et al. 2007). Well known xylose fermenting yeasts such as *Pichia stipitis* (Toivola et al. 1984), *Candida shehatae* (Chandel et al. 2007; Palnitkar and Lachke 1992), and *Pachysolen tannophilus* (Bolen and Detroy 1985) have been studied for decades. These ethanol producing xylose fermenting yeasts metabolize xylose through XR-XDH pathway (Figure 1.2). Many XRs are NADPH-dependent (Matsushika et al. 2009) and XRs from *P. stipitis* or *P. tannophilus* possess dual specificities for both NAD(P)H but with the preference of NADPH (Bicho et al. 1988; Verduyn et al. 1985). For example, in different *P. stipitis* strains, the  $K_m$  of xylose on NADH is 3 to 15 folds higher than  $K_m$  of xylose on NADPH (van Vleet and Jeffries 2009; Verduyn et al. 1985). XDHs are normally NAD<sup>+</sup>-dependent (Hahn-Hägerdal et al. 2007). Therefore the amount of NAD<sup>+</sup> required by XDH may not be satisfied since XR prefers to reoxidize NADPH rather than NADH. The insufficient amount of NAD<sup>+</sup> will then lead to a block of the XR-XDH pathway and result in xylitol accumulation (Figure 1.2). Under aerobic conditions, the surplus NADH can be reoxidized by oxygen (Nevoigt 2008). In order to solve the problem of xylitol accumulation under anaerobic conditions, a carefully controlled microaerophilic condition is

often employed for xylose fermentation. The oxygen has to be precisely controlled so that the amount of oxygen is only used as electron acceptor for surplus NADH but not interfering with the other metabolism of yeast. Studies on optimizing the oxygen flow rate in order to control xylitol-formation/biomass-production and optimize ethanol yield/productivity have been performed (Fonseca et al. 2007; Skoog and Hahn-Hägerdal 1990). Nevertheless, the requirement of precisely controlling air flow is both economically heavy and technically difficult to conduct in large-scale industrial conditions (Hahn-Hägerdal et al. 2007), especially during the fermentation of high dry matter lignocellulose where the introduction of air into the medium is uneven. Yeast *S. passalidarum* was discovered as harbouring NADH preferred XR and NAD<sup>+</sup>-dependent XDH (Chapter 2). This property solved the problem of cofactor imbalance under the anaerobic conditions and resulted in a perfect anaerobic fermentation of xylose (Chapter 2). In addition, Hahn-Hägerdal et al. (2007) stated that “anaerobic growth reflects cell viability and is thus a desirable quality in industrial fermentation. Neither natural nor recombinant xylose-metabolizing yeast grow anaerobically on xylose, the molecular reasons for which are not entirely known.” *S. passalidarum*, however, showed a rapid and efficient anaerobic growth on xylose (Chapter 2) and therefore this xylose fermenting yeast is a promising candidate for industrial ethanol production. Unfortunately, this yeast also showed quite low tolerance towards inhibitors present in the pretreated lignocellulosic biomass (Chapter 3 and Chapter 5), which prevents the immediate application of this yeast in industrial fermentation. The sensitivity to inhibitors in the lignocellulosic material is a common problem for the naturally xylose-fermenting yeasts.

#### **1.3.2.1.4 Recombinant xylose fermenting yeast**

As stated above, most naturally xylose fermenting yeasts require expensive and difficult microaerophilic conditions and have low inhibitor tolerance. Traditional baker's yeast *S. cerevisiae*

is recognised as the most robust and suitable host for ethanol production from glucose (Almeida et al. 2009; Rumbold et al. 2009), however it lacks the ability to ferment xylose. Therefore, during the past decades, efforts have been made to introduce the capacity of xylose fermentation into *S. cerevisiae* (Nevoigt 2008). A number of recombinant xylose fermenting *S. cerevisiae* strains have been constructed by introducing XR and XDH from *Pichia stipitis* (Matsushika et al. 2009; Ho et al. 1998) or XI isomerase from the fungi *Piromyces* (Kuyper et al. 2005) and *Orpinomyces* (Tanino et al. 2010). However, the cofactor imbalance between XR and XDH also exists in these recombinant *S. cerevisiae* carrying exogenous *Ps* XRs and XDHs, resulting in xylitol accumulation and incomplete xylose fermentation. In order to solve these problems, manipulation of coenzyme preference have been tried in some studies, e.g. altering the coenzyme preference of XR to favour NADH by structure-guided site-directed mutagenesis (Bengtsson et al. 2009; Petschacher and Nidetzky 2008; Jeppsson et al. 2006) or altering the coenzyme preference of XDH into NADP<sup>+</sup> by site-directed mutagenesis (Watanabe et al. 2007). However, although these studies to some extent have succeeded in improving the ethanol production from xylose, the approach is limited by the rigidity of the central metabolism in *S. cerevisiae*. In comparison, introduction of XI pathway avoids the problem of cofactor imbalance. But it is difficult to conclude that the XI pathway is superior to XR-XDH pathway, as *S. cerevisiae* strain engineered with the XR-XDH pathway showed much higher aerobic growth rate and anaerobic xylose consumption rate than the strain engineered with the XI pathway (Karhumaa et al. 2007). The discovery of the naturally NADH preferred XR in *Spathaspora passalidarum* (Chapter 2) provide an alternative solution to the cofactor imbalance problem.

Another common problem with the recombinant xylose fermenting *S. cerevisiae* is the lack of efficient xylose transport system, which affects the rate of xylose utilization. Expressing glucose/xylose facilitated transporter (Gxf1) from *Candida intermedia* enhanced the growth rate of

a recombinant *S. cerevisiae* especially when the xylose concentration was low (Runquist et al. 2009). The growth on xylose of recombinant xylose fermenting *S. cerevisiae* strains were significantly improved by heterologous expressing a putative xylose specific transporter from *Trichoderma reesei* (Saloheimo et al. 2007) or two xylose transporters from *Arabidopsis thaliana* (Hector et al. 2008).

Finally, although there is progress in constructing recombinant xylose fermenting yeast, all the engineered laboratory strains are still inferior to industrial *S. cerevisiae* strains when fermenting the real lignocellulosic hydrolysate (van Vleet and Jeffries 2009; Hahn-Hägerdal et al. 2007).

### **1.3.2.2 Inhibition mechanism and inhibitor tolerance**

Many industrial *S. cerevisiae* strains with higher tolerance towards lignocellulosic hydrolysate have been isolated (Hahn-Hägerdal et al. 2007). However the inhibitor tolerance of these strains still require improvement, especially during the high dry matter fermentation where the inhibitors are present at high level (Rudolf et al. 2005). Naturally xylose fermenting yeasts are generally inhibited by industrial lignocellulosic substrates (Chapter 5; Hahn-Hägerdal et al. 2007; Klinke et al. 2004). Recombinant xylose fermenting *S. cerevisiae* strains made in an industrial yeast genetic background have been constructed in many labs (Lau and Dale 2009; Katahira et al. 2006; Sedlak and Ho 2004), but they still showed problems of either inefficient xylose fermentation or inferior inhibitor tolerance as compared to the parental strains.

#### **1.3.2.2.1 Inhibition mechanism**

Pretreatment generated inhibitors generally can be categorized into furans, weak acids, and phenolic compounds (Section 1.2.2.1). The mechanisms of inhibition have been studied extensively during the past decades, but still they have not been elucidated.

## *Furans*

The aldehydes furfural and HMF are two of the main inhibitors present in the lignocellulosic material. Furfural normally appears to be more toxic than HMF (Chapter 3; Palmqvist and Hahn-Hägerdal 2000b). The toxicity of furfural is directly correlated with the toxicity of lignocellulosic materials (Heer and Sauer 2008). Furfural and HMF cause the reduction of volumetric ethanol yield and productivity, inhibit cell growth and give rise to lag phase. The extent of the deleterious effects depends on the concentration of furans and the yeast strain. Big variations of inhibition effects caused by furans can be found with different yeast strains, even within the same species of *S. cerevisiae* (Almeida et al. 2007). Furfural and HMF shows high toxicity to the xylose fermenting yeast *S. passalidarum*, as 0.5 g/L and 1 g/L, respectively, can cause around 50% inhibition of cell growth and severe inhibition on sugar consumption and ethanol production (Chapter 3). The mechanism of furan inhibition is very complex, with regulations of numerous metabolic pathways and gene expressions involved. For example, *in vitro* measurements showed that furfural and HMF inhibited the activity of alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH) (Modig et al. 2002). Furfural affected central carbon metabolism of cells such as pentose phosphate pathway (Gorsich et al. 2006), glycolytic pathway (Palmqvist et al. 1999) and tricarboxylic acid cycle (TCA) (Sarvari Horvath et al. 2003). Furfural may cause intracellular NAD(P)H depletion, as suggested by the decreased glycerol formation (Chapter 3; Palmqvist and Hahn-Hägerdal 2000b) or increased levels of acetaldehyde secretion in the presence of furfural (Chapter 3; Palmqvist et al. 1999). Furfural and HMF also affect the regulations of genes involved in DNA damage repair, nutrient starvation and osmotic stress (Lin et al. 2009), indicating cell damages.

## *Weak acids*

Acetic acid, formic acid and levulinic acid are the most common weak acids present in lignocellulosic hydrolysate (Palmqvist and Hahn-Hägerdal 2000b). Weak acids are normally less toxic than furans and phenolic compounds to yeast (Delgenes et al. 1996). The inhibition mechanism of weak acids is proposed as cytoplasmic acidification and ATP depletion. Undissociated weak acid enters across the cell membrane by diffusion and is dissociated to  $H^+$  and anion due to the higher intracellular pH (Russell 1992). The anionic form of the acid is captured inside the cell and the undissociated acid continuously enters into the cell until equilibrium is reached. Thus, the cytosol is gradually acidified by the increasing amount of  $H^+$  accompanied by accumulation of the intracellular anions. This acidified intracellular environment will cause severe problems to intracellular reactions. In order to increase the intracellular pH,  $H^+$  is pumped out by the plasma membrane ATPase at the expense of 1 mol ATP per 1 mol of  $H^+$ . Consequently, less ATP is available for cell metabolism and ATP depletion may happen when the weak acid concentration in the medium is high. Differences in the toxicity among the above three weak acids has been reported (Larsson et al. 1999a), and they may be explained by differences in cell membrane permeability or in degree of dissociation at the same internal pH.

#### *Phenolic compounds*

Phenolic compounds are considered to be the most toxic compounds. Low molecular weight (MW) phenolic compounds are more inhibitory to *S. cerevisiae* than high MW phenolic compounds (Klinke et al. 2004). The inhibition mechanisms of phenolic compounds are the least understood, largely due to the diversity of the group and the trace amount dosage of each compound (Palmqvist and Hahn-Hägerdal 2000b). Model studies of the inhibitory effects of phenolic compounds have been performed at concentrations far higher than those actually present in the lignocellulose hydrolysate, due to the lack of accurate quantitative analysis methods (Palmqvist and Hahn-Hägerdal 2000b). One possible inhibition mechanism of phenolic compounds could be the

disturbance of the cell membrane integrity caused by the binding of phenolic compounds with cell membrane structure, affecting the cell membrane's function as selective barriers and enzyme matrices (Heipieper et al. 1994).

#### *Synergistic effects*

Studies have reported the synergistic effects among the inhibitors. The combination of inhibitors leads to more inhibition than the linear sum of the inhibition effects caused by each inhibitor. This has been demonstrated for combinations of furfural and HMF (Liu et al. 2004), furfural and acetic acid (Palmqvist and Hahn-Hägerdal 2000b) and furfural/HMF together with a variety of phenolic and aromatic compounds (Mussatto and Roberto 2004; Delgenes et al. 1996). Furfural-resistant mutants of *S. passalidarum* can tolerate furfural concentration at 2 g/L in the synthetic medium but cannot survive in the liquid fraction of pretreated wheat straw hydrolysate where the furfural concentration is only around 0.4 g/L (Chapter 4), indicating that the synergistic effects of a variety of inhibitors contribute to the inhibition in lignocellulosic hydrolysate.

#### **1.3.2.2.2 Inhibitor tolerance and strain improvement**

*S. cerevisiae* is able to convert furfural and HMF into less toxic compounds. Furfural is converted to furfuryl alcohol (i.e. 2-hydroxymethylfuran) (Palmqvist et al. 1999) and HMF to HMF alcohol (i.e. 2,5-bis-hydroxymethylfuran) (Liu et al. 2004) under anaerobic conditions. Furfural can also be oxidized into furoic acid by *S. cerevisiae* under aerobic conditions (Taherzadeh et al. 1999). The similar situation was found in xylose-fermenting yeast such as *P. stipitis* (Liu et al. 2004) and *S. passalidarum* (Chapter 3). Furfuryl alcohol cause only slight inhibition on anaerobic growth of yeast such as *S. cerevisiae* (Palmqvist et al. 1999) and *S. passalidarum* (Chapter 3). This reduction of furfural and HMF requires NADH or NADPH, depending on different strains. In most *S. cerevisiae* strains, furfural reduction is NADH dependent and HMF reduction is NADPH dependent



(Almeida et al. 2007). Only one *S. cerevisiae* strain ATCC 96581 (=TMB3000) has been reported to be NADH-dependent for HMF reduction (Nilsson et al. 2005). Both furfural and HMF reduction was found to be NADH-dependent in *S. passalidarum* (Chapter 3). Studies demonstrated many reductases such as alcohol dehydrogenases ADH6 and ADH7 having the ability to catalyze furfural and HMF reductions (Heer et al. 2009; Petersson et al. 2006; Larroy et al. 2002a; Larroy et al. 2002b). Overexpression of the genes of these reductases resulted in the increase of both *in vivo* and *in vitro* furan conversion rates. *P. stipitis* xylose reductase (*Ps* XR) has recently been reported to harbour the dual capacity for catalyzing both xylose reduction and HMF reduction. Overexpression of *Ps* XR increased *in vivo* HMF conversion rate by 20% (Almeida et al. 2008). *S. passalidarum* XR may also have such dual functions. Slight improvement of fermentation performance on furfural/HMF by *S. passalidarum* with the presence of xylose was observed, and *in vitro* measurements showed a significant increase in the activity of both xylose reduction and furfural/HMF reduction using extracts of cells incubated by xylose (Chapter 3). Moreover, the NAD(P)H requirement of furan reduction suggests another way of improving the furan tolerance, via enlarging the intracellular pool of NAD(P)H. For example, by overexpressing genes of *ZWF1*, encoding glucose-6-phosphate dehydrogenase, the flux through PPP pathway was increased, and therefore more NADPH was available. The engineered strain showed the capacity of growing at higher furfural concentrations (Gorsich et al. 2006). Other metabolic engineering work aiming at improving the strain's inhibitor tolerance towards phenolic compounds have also been tried, e.g. inserting genes of enzyme laccase, acting on removing phenol compounds, from *Trametes versicolor* into *S. cerevisiae* (Larsson et al. 2001a) and overexpressing *S. cerevisiae* PAD1 encoding phenolic acid carboxylase (Larsson et al. 2001b).

However, even the tolerance to a single compound e.g. HMF can correlate with hundreds of genes (Ma and Liu 2010), not to mention the tolerance to the multitude inhibitors present in the

lignocellulosic hydrolysate. Moreover, metabolic networks underlying the inhibitor tolerance are still unclear and the knowledge of genome sequence of many yeasts such as *S. passalidarum* (this study) is still lacking. In this context, rational metabolic engineering approaches can be extremely difficult. Therefore alternative approaches such as evolutionary engineering and genome shuffling can be necessary to address the challenge (Petri and Schmidt-Dannert 2004). Evolutionary engineering is based on the adaption process with directed selection pressure, mimicking the natural evolution for the selection of desired phenotypes. Genome shuffling is based on the technique of mutagenesis and protoplast fusion aiming at randomly shuffled genes, followed by a directed selection. This method provides advantages of multiple changes throughout the entire genome without requiring the knowledge of genome sequence and metabolic network information, and high efficiency of gene transfer and recombination can be achieved by fusing complete protoplast genomes. Multiple inhibitor tolerance towards lignocellulosic substrates of *S. passalidarum* and *S. cerevisiae* strains were improved through the methods of genome shuffling (i.e. mutagenesis and protoplast fusion) (Chapter 4) and evolutionary engineering (i.e. directed adaptation) (Chapter 5), respectively. The above approaches offer a possibility of rapidly improving the desired phenotype of strains when the necessary knowledge for metabolic engineering is lacking. Moreover, the legislation on gene modified organisms (GMO) are still against the industrial application of recombinant yeast strains in many countries, but strains manipulated by evolutionary engineering and genome shuffling are not considered as GMO so that they can be directly applied in industry.

#### **1.4 Focus of dissertation**

The research work presented in this dissertation aimed at improving the cost efficiency of the fermentation step in the process of lignocellulosic ethanol production. The efforts were mainly on:

- (1) physiology and mechanism study of anaerobic xylose fermentation and inhibitor tolerance of a

novel xylose fermenting yeast *S. passalidarum* (Chapter 2 and Chapter 3); (2) construction of yeast strains with capacity of xylose fermentation under complete anaerobic conditions and improved inhibitor tolerance (Chapter 4); and (3) test of the improved strains in the real lignocellulosic context (Chapter 5).

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# Chapter 2

## Anaerobic xylose fermentation by *Spathaspora passalidarum*

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### 2.1 Abstract

A cost-effective conversion of lignocellulosic biomass into bioethanol requires that the xylose released from the hemicellulose fraction (20-35% of biomass) can be fermented. Baker's yeast, *Saccharomyces cerevisiae*, efficiently ferments glucose but it lacks the ability to ferment xylose. Xylose-fermenting yeast such as *Pichia stipitis* requires accurately controlled microaerophilic conditions during the xylose fermentation, rendering the process technically difficult and expensive. In this study it is demonstrated that under anaerobic conditions *Spathaspora passalidarum* showed high ethanol production yield, fast cell growth and rapid sugar consumption with xylose being consumed after glucose depletion, while *Pichia stipitis* was almost unable to utilize xylose under the same condition. It is further demonstrated that for *S. passalidarum* the xylose conversion takes place by means of NADH-preferred xylose reductase (XR) and NAD<sup>+</sup>-dependent xylitol dehydrogenase (XDH). Thus, the capacity of *S. passalidarum* to utilize xylose under anaerobic conditions is possibly due to the balance between the cofactor's supply and demand through this XR-XDH pathway. Only one other XR with NADH preference have been reported so far. 2-deoxy glucose completely inhibited the conversion of xylose by *S. passalidarum* under anaerobic conditions, but only partially did that under aerobic conditions. Thus, xylose uptake by *S. passalidarum* may be carried out by different xylose transport systems under anaerobic and aerobic conditions. The presence of glucose also repressed the enzymatic activity of XR and XDH from *S. passalidarum* as well as the activities of those enzymes from *P. stipitis*.

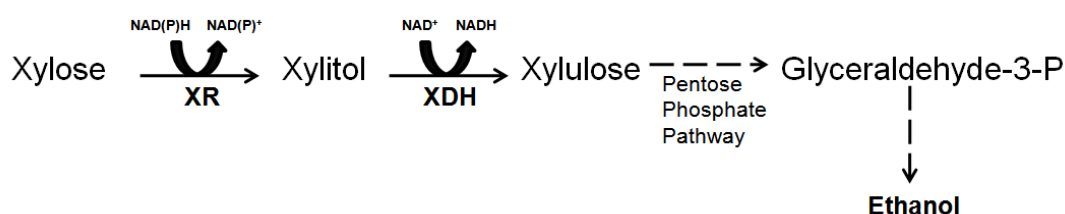
## 2.2 Introduction

Bioethanol, considered as a clean liquid fuel, has a potential as a substitute for liquid fossil fuel which is regarded as one of the main contributors to global-warming. Bioethanol has been produced by fermentation with baker's yeast of sugars from crops such as sugar cane and corn starch for many years in countries all over the world. The total global annual production reached 23 billions of gallons in 2010 (Renewable Fuels Association 2011). However, use of agricultural crops for bioethanol production competes with food production, creating a demand for technologies which allow bioethanol production from non-food plants and lignocellulosic biomass residues such as grass, straw, and wood chips. Lignocellulosic biomass comprises 35-50% cellulose and 20-35% hemicellulose (Saha 2003) which, after enzymatic hydrolysis, can be converted into glucose and mainly xylose, respectively. However, with traditional baker's yeast *Saccharomyces cerevisiae* only the glucose can be fermented into ethanol and this makes ethanol production from lignocellulosic biomass less economical. Other microorganisms are able to produce ethanol by fermenting xylose, such as anaerobic bacteria and filamentous fungi, but these microorganisms have much lower ethanol tolerance and most of the ethanol-producing bacteria convert sugars to mixed products where ethanol just comprises a minor part (Wiegel et al. 1983) while filamentous fungi convert sugars at extremely low rates (Hahn-Hägerdal et al. 2007).

Yeasts such as *Pichia stipitis* and *Candida shehatae* are able to ferment xylose into ethanol and they have been studied for decades (Agbogbo and wenger 2007; Delgenes et al. 1996). Recombinant *Saccharomyces* strains carrying an exogenous xylose utilization pathway have also been constructed in many laboratories (Kuyper et al. 2005; Ho et al. 1998). In most of these xylose fermenting yeasts, xylose is metabolized by (1) reduction of xylose to xylitol by the xylose reductase (XR) and (2) oxidization of xylitol to xylulose by xylitol dehydrogenase (XDH). Subsequently xylulose enters into the pentose phosphate pathway being converted to

glyceraldehyde-3-P which is further reduced to ethanol (Figure 2.1). Most XRs (e.g. from *P. stipitis* or *P. tannophilus*) possess dual specificities for both NADPH and NADH, but with the preference of NADPH (Bicho et al. 1988; Verduyn et al. 1985). Most XDHs utilize  $\text{NAD}^+$  as cofactor. Xylitol will be accumulated if insufficient amounts of  $\text{NAD}^+$  are regenerated, and the xylose metabolism will therefore be blocked (Hahn-Hägerdal et al. 2007; Skoog and Hahn-Hägerdal 1990). In order to solve such a problem, the presence of very low but accurately controlled oxygen during the xylose fermentation is needed to regenerate the required  $\text{NAD}^+$  from NADH (Skoog and Hahn-Hägerdal 1990; van Maris et al. 2007). However, such accurate control of oxygen in large-scale industrial processes, especially when viscous lignocellulosic hydrolysates are used as a source of fermentable sugars, is technically difficult and expensive (Hahn-Hägerdal et al. 2007). Therefore microorganism that can convert xylose into ethanol under anaerobic conditions is a prerequisite for a cost-effective lignocellulosic ethanol production. Furthermore, the large investments in production facilities for bioethanol production developed for *S. cerevisiae* makes it essential that the xylose-fermenting microorganism selected for this purpose should be compatible with these facilities.

This study shows that the wood-boring-beetle associated yeast, *Spathaspora passalidarum* (Nguyen et al. 2006), can convert xylose into ethanol with much higher efficiency than *Pichia stipitis* under anaerobic conditions, suggesting that it may be a promising candidate strain for industrial fermentation of lignocellulosic hydrolysate. The mechanism of the efficient anaerobic xylose fermentation by this yeast is also investigated.



**Figure 2.1** Xylose fermentation pathway in xylose-fermenting yeast; XR: xylose reductase; XDH: xylitol dehydrogenase

## 2.3 Materials and Methods

### *Media*

The culture and the selection media used in this study are listed below: (1) Yeast extract peptone dextrose (YPD) medium: 10 g/L yeast extract, 20 g/L peptone and 30 g/L glucose; (2) Half defined (HD) medium: 5 g/L peptone, 5 g/L yeast extract, 1 g/L  $\text{KH}_2\text{PO}_4$ , 2 g/L  $\text{NH}_4\text{Cl}$ , 0.3 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and glucose and/or xylose as indicated.

### *Yeast cultivations*

Yeast strains *Pichia stipitis* ATCC 58376 and *Spathaspora passalidarum* ATCC MYA-4345 were used in this study. *P. stipitis* ATCC 58376 is a xylose-fermenting yeast (Toivola et al. 1984). *S. passalidarum* is a recently discovered xylose-fermenting yeast associated with wood-boring beetles (Nguyen et al. 2006). Both strains were from the American Type Culture Collection (ATCC). The yeast strains were propagated in YPD medium by inoculating single colonies isolated from YPD agar plate. Pure yeast cultures were stored at  $-80^\circ\text{C}$  with addition of 20% glycerol. Pre-cultures were prepared by inoculating frozen cells into 40 ml YPD medium in 100 ml cotton plugged conical flasks. The flasks were incubated at  $32^\circ\text{C}$  in an orbital shaker operating at 120 rpm for 16-18 hours.

### *Anaerobic fermentation and aerobic growth*

All anaerobic fermentations were performed as batch fermentation with 50 ml working volume in 100 ml anaerobic vials. Residual oxygen in the medium and in head space of the vial was not flushed out in order to support initial cell growth. The vials were inoculated with a pre-culture to a density at  $\text{OD}_{620\text{nm}}$  of 0.05~0.1. The vials were incubated at  $32^\circ\text{C}$  in an orbital shaker with rotation speed of 120 rpm. Samples were taken under sterile conditions at certain time intervals.

Aerobic growth experiments were performed with 50 ml working volume in 100 ml cotton plugged conical flasks. The inoculum concentration was at  $OD_{620nm}=0.05$ . The flasks were incubated at 32°C in an orbital shaker with rotation speed of 120 rpm. Samples were taken out under sterile conditions at certain time intervals.

### ***Fermentation analysis***

During the fermentation, the cell growth was monitored by measuring the optical density at 620nm using Ultrospec 3000 (Pharmacia Biotech, Sweden). After the fermentation, the dry weight of the cell biomass was estimated by filtering through a membrane filter (0.45 µm) and drying the filter overnight at 105°C.

Values of the maximum specific growth rate ( $\mu_{max}$ ) were calculated by the slope of the straight line from the plot of  $\ln OD_{620nm}$  versus time. R-squared values of the linear regressions were in the range of  $0.98 \pm 0.01$ .

Carbon recovery was calculated based on carbon yields in the synthesized cell biomass and the organic products plus stoichiometrically associated CO<sub>2</sub> production that accompanied the production of ethanol and acetic acid. The yeast cell biomass composition was assumed to be CH<sub>1.8</sub>O<sub>0.5</sub>N<sub>0.2</sub> (Verduyn et al. 1990). The formula used is the following:

*% Carbon recovery =*

$$\frac{3 \bullet (\text{mM ethanol} + \text{mM acetate}) + 5 \bullet \text{mM xylitol} + 3 \bullet \text{mM glycerol} + \text{mM synthesized cell biomass}}{5 \bullet \text{mM pentose} + 6 \bullet \text{mM hexose}}$$

Carbon recovery has been calculated for all the fermentations in this study and all the values are in the range of 95 -105%.

Glucose, xylose, ethanol, xylitol, glycerol, and acetic acid were analyzed by a high performance liquid chromatography (HPLC) system (Shimadzu, Japan) using refractive index detector equipped

with an Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) running at 63°C with 4 mM H<sub>2</sub>SO<sub>4</sub> as eluent with a flow rate of 0.6 ml/min.

#### ***Enzymatic activity assay of xylose reductase and xylitol dehydrogenase***

For *in vitro* enzymatic activity assay of xylose reductase (XR) and xylitol dehydrogenase (XDH), *S. passalidarum* and *P. stipitis* were grown overnight in HD medium with 30 g/L glucose and 30 g/L xylose. Cells were harvested from 10 ml culture volume by centrifugation at 4500 rpm for 10 minutes at 4°C. The cell pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.0), and the pellet was re-suspended in 900 µl of the same buffer with the addition of 100 µl acid-washed glass beads (particle size: 425-600 µm, U.S. Sieve). Cell disruption was conducted by using a Bullet Blender (Next Advance Inc., USA) at 4°C. The final homogenates were centrifuged at 14000 rpm for 5 minutes at 2°C. The supernatants were used for enzymatic activity assay. Protein concentrations in the cell-free extract were determined by “Quick Start™ Bradford Protein Assay Kit” (Bio-Rad Laboratories Ltd., USA) following the manufacture’s instruction.

Enzymatic activities were determined spectrophotometrically by following the oxidation or reduction of the coenzymes at 340 nm. Dynamic measurement of the absorbance at 340 nm ( $A_{340\text{nm}}$ ) was carried out by Lambda 25 UV/VIS spectrometer (PerkinElmer, USA) at 25°C, with an interval time of 5 seconds for recording and a total measuring time of 3 minutes for each reaction. Enzyme solutions were diluted if necessary to ensure a constancy of  $\Delta(A_{340\text{nm}})/\Delta(\text{time})$  lasting for at least 1 minute.

Apparent kinetic parameters of XR for xylose reduction were obtained from initial-rate data recorded at the concentration of NAD(P)H at 400 µM. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0; 0.4 mM NAD(P)H; 50 mM D-xylose; cell-free extract; and distilled water in a total volume of 400 µl.



The Michaelis-Menten constants for XR on NADPH and NADH ( $K_m(\text{NADPH})$  and  $K_m(\text{NADH})$ ) were determined from the initial-rate data measured at saturated xylose concentration (500 mM) with six concentrations of NADPH (25-500  $\mu\text{M}$ ) and NADH (25-500  $\mu\text{M}$ ), respectively. The Michaelis-Menten constant for XR on xylose ( $K_m(\text{xylose})_{\text{NADPH}}$  and  $K_m(\text{xylose})_{\text{NADH}}$ ) were determined from the initial-rate data measured at saturated level of NADPH (400  $\mu\text{M}$ ) and NADH (400  $\mu\text{M}$ ), respectively, with six concentrations of xylose (20-500 mM).

Apparent kinetic parameters of XDH for xylitol oxidation were obtained from initial-rate data recorded at the concentration of  $\text{NAD(P)}^+$  at 400  $\mu\text{M}$ . The reaction mixture contained 25 mM potassium carbonate buffer pH 9.5; 0.4 mM  $\text{NAD(P)}^+$ ; 20 mM xylitol; cell-free extract; and distilled water in a total volume of 400  $\mu\text{l}$ .

The Michaelis-Menten constants for XDH on  $\text{NADP}^+$  and  $\text{NAD}^+$  ( $K_m(\text{NADP}^+)$  and  $K_m(\text{NAD}^+)$ ) were determined from the initial-rate data measured at saturated xylitol concentration (400 mM) with six concentrations of  $\text{NADP}^+$  (25–500  $\mu\text{M}$ ) and  $\text{NAD}^+$  (25-500  $\mu\text{M}$ ), respectively. The Michaelis-Menten constant for XDH on xylitol ( $K_m(\text{xylitol})_{\text{NADP}^+}$  and  $K_m(\text{xylitol})_{\text{NAD}^+}$ ) were determined from the initial-rate data measured at saturated level of  $\text{NADP}^+$  (400  $\mu\text{M}$ ) and  $\text{NAD}^+$  (400  $\mu\text{M}$ ), respectively, with six concentrations of xylitol (10-400 mM).

## 2.4 Results

### *Fermentation and growth performance of S. passalidarum*

*S. passalidarum* is able to use xylose as the sole carbon source for biomass growth and ethanol production under complete anaerobic conditions (Figure 2.2B). The final ethanol production from glucose (30 g/L), xylose (32 g/L) and a mixture of glucose and xylose (15 g/L glucose and 15 g/L xylose) were comparable (Figure 2.2A, 2.2B and 2.2C), and all of the ethanol yields exceeded 90% of the theoretical ethanol yield. The maximum specific growth rates of *S. passalidarum* in these

three cases are listed in Table 2.1. *S. passalidarum* grew slightly slower on xylose than on glucose. The final biomass (as expressed as OD<sub>620nm</sub>) with xylose is less than that with glucose (Figure 2.2A and 2.2B). The correlation between cell number and OD<sub>620nm</sub> of *S. passalidarum* was determined in HD medium with 30 g/L glucose (HDD) and in HD medium with 30 g/L xylose (HDX). In both cases the correlation was linear and essentially identical (Appendix Figure A1) with OD<sub>620nm</sub>=1 corresponding to approximately  $1 \times 10^7$  cells/ml.

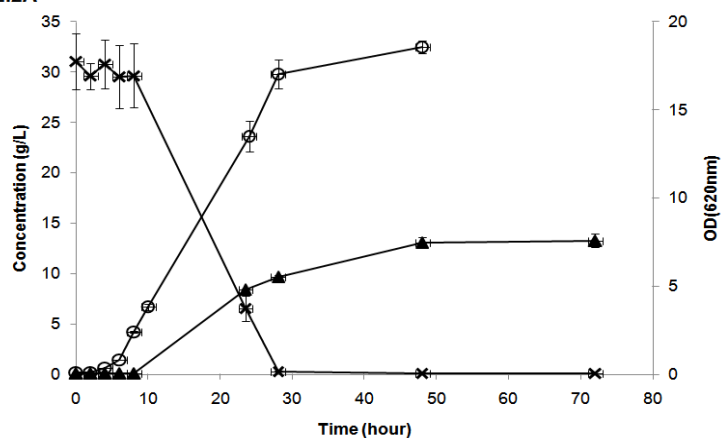
Under anaerobic conditions *S. passalidarum* consumed xylose after glucose (Figure 2.2C and Figure 2.3A), while under aerobic conditions glucose and xylose were consumed simultaneously (Figure 2.3C). In comparison, the other xylose-fermenting yeast *P. stipitis* hardly utilize xylose under anaerobic conditions (Figure 2.3B) and only 9% of the total amount of xylose was consumed after 124 hours (data not shown). Under aerobic conditions, *P. stipitis* displayed sequential sugar consumption, first utilizing glucose and then xylose (Figure 2.3D). Thus, *S. passalidarum* and *P. stipitis* are different in their capacity to utilize glucose and xylose under aerobic and anaerobic conditions.

**Table 2.1** Maximum specific growth rate of *S. passalidarum* on different carbon source under anaerobic conditions<sup>a</sup>

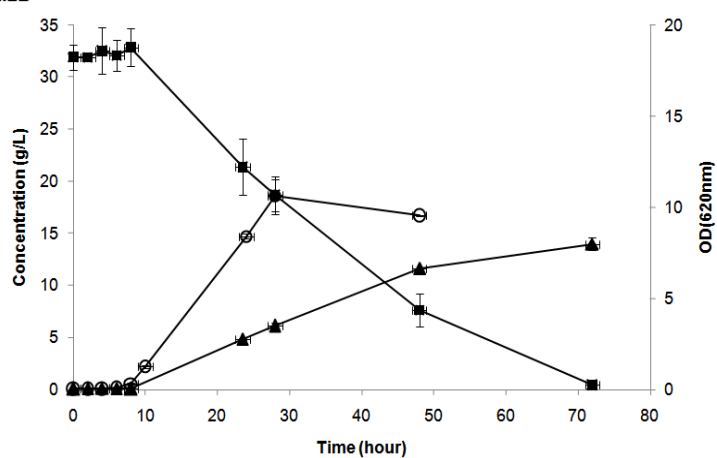
Carbon source	30 g/L Glucose	30 g/L Xylose	15 g/L Glucose + 15 g/L Xylose	30 g/L Glucose + 30 g/L Xylose
$\mu_{\max}$ (h <sup>-1</sup> )	0.67 ± 0.04	0.58 ± 0.04	0.63 ± 0.05	0.66 ± 0.00

<sup>a</sup> Data presented are average of triplicate experiments ± standard deviations

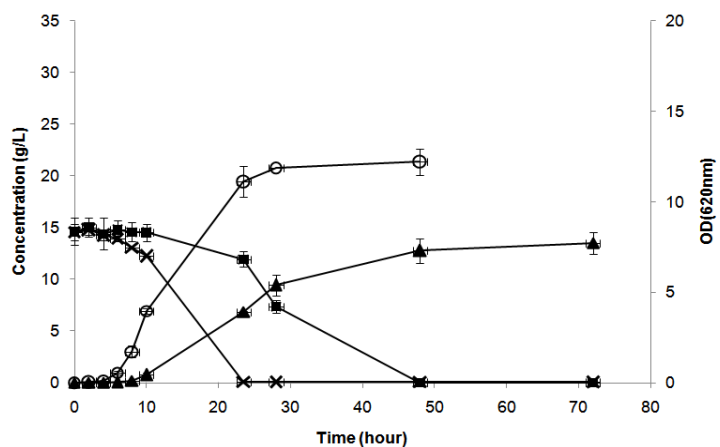
### 2.2A



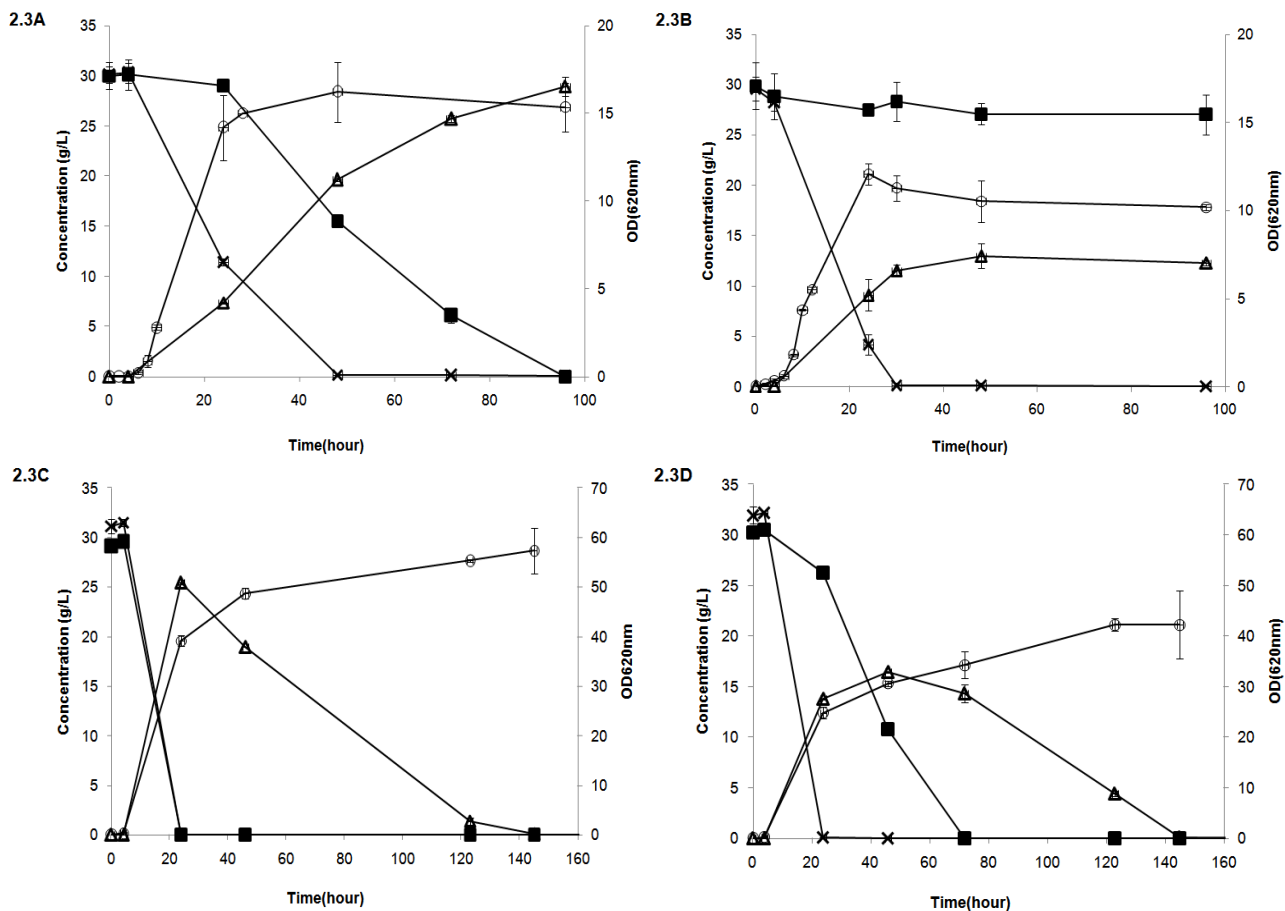
### 2.2B



### 2.2C



**Figure 2.2** Sugar fermentation and cell growth of *S. passalidarum* under anaerobic conditions in HD medium with addition of 30 g/L glucose (**2.2A**) or 32 g/L xylose (**2.2B**) or 15 g/L glucose + 15 g/L xylose (**2.2C**); glucose (x), xylose (filled square), ethanol (filled triangle), biomass OD (open circle). Data presented are average of triplicate experiments and error bars indicate the standard deviations.



**Figure 2.3** Sugar consumption in HD medium with 30 g/L glucose and 30 g/L xylose by *S. passalidarum* (2.3A: under anaerobic conditions; 2.3C: under aerobic conditions) and *P. stipitis* (2.3B: under anaerobic conditions; 2.3D: under aerobic conditions); glucose (x), xylose (filled square), ethanol (open triangle), biomass OD (open circle). Data presented are average of triplicate experiments and error bars indicate the standard deviations.

### Co-factor preference of xylose reductase and xylitol dehydrogenase

*S. passalidarum* produced xylitol at a much lower yield than *P. stipitis* under anaerobic conditions (Table 2.2). *S. passalidarum* completely consumed 30 g/L xylose and only formed xylitol at a yield of 0.09 g/g consumed xylose. *P. stipitis* only consumed 9% of xylose but formed xylitol at a yield of 0.2 g/g consumed xylose. The data from a recombinant *Saccharomyces cerevisiae* strain RWB217 with xylose isomerase expressed (Kuyper et al. 2005) is also included in Table 2.2 as an external reference. The xylitol yield by this recombinant strain was almost negligible (0.006 g/g consumed xylose), since the xylose isomerase catalyzes the direct conversion of xylose to xylulose without xylitol formation.

The kinetic constants of XR and XDH from *S. passalidarum* and *P. stipitis* were determined and the values are presented in Table 2.3. *P. stipitis* XR showed a higher  $K_m$  value on NADH ( $20.9 \pm 5.5 \mu\text{M}$ ) than on NADPH ( $13.3 \pm 1.4 \mu\text{M}$ ), which is in accordance with previously published values (Verduyn et al. 1990). *S. passalidarum* XR showed a significantly lower  $K_m$  on NADH ( $17.3 \pm 1.8 \mu\text{M}$ ) than on NADPH ( $31.7 \pm 2.6 \mu\text{M}$ ). When determining  $K_m$  value on xylose, *S. passalidarum* XR also strongly preferred NADH ( $K_m(\text{xylose})_{\text{NADH}} = 17.1 \pm 1.8 \text{ mM}$ ) over NADPH ( $K_m(\text{xylose})_{\text{NADPH}} = 52.2 \pm 3.8 \text{ mM}$ ). Both *S. passalidarum* XDH and *P. stipitis* XDH exclusively used  $\text{NAD}^+$  as the cofactor.

**Table 2.2** Xylitol yield of different yeast strains during anaerobic batch fermentations

Microorganism	Xylitol yield (g/g-consumed xylose)	Medium
<i>S. passalidarum</i> <sup>a</sup>	$0.09 \pm 0.00$	HD+30 g/L glucose+30 g/L xylose
<i>P. stipitis</i> <sup>b</sup>	$0.22 \pm 0.03$	HD+30 g/L glucose+30 g/L xylose
<i>Saccharomyces cerevisiae</i> strain RWB217 with xylose isomerase expressed <sup>c</sup>	$0.006 \pm 0.000$	Synthetic medium as described by Kuyper et al. (2005) with 20 g/L glucose + 20 g/L xylose

<sup>a</sup> *S. passalidarum* consumed all the xylose in the medium during the anaerobic batch fermentation

<sup>b</sup> *P. Stipitis* only consumed 9% of the xylose in the medium during the anaerobic batch fermentation

<sup>a+b</sup> Data presented are average of triplicate experiments  $\pm$  standard deviations

<sup>c</sup> Calculated from the data presented by Kuyper et al. (2005)

**Table 2.3** Kinetic parameters of xylose reductase and xylitol dehydrogenase from crude cell extracts of *S. passalidarum* and *P. stipitis*<sup>a</sup>

Strain	Enzyme	Cofactor	$K_{m1} (\mu\text{M})$ <sup>b</sup>	$K_{m2} (\text{mM})$ <sup>c</sup>
<i>S. passalidarum</i>	Xylose reductase (XR)	NADH	$17.3 \pm 1.8$	$17.1 \pm 1.8$
		NADPH	$31.7 \pm 2.6$	$52.2 \pm 3.8$
	Xylitol dehydrogenase (XDH)	$\text{NAD}^+$	$39.2 \pm 0.8$	$4.8 \pm 0.7$
		$\text{NADP}^+$	-	-
<i>P. stipitis</i> ATCC 58376	Xylose reductase (XR)	NADH	$20.9 \pm 5.5$	$80.3 \pm 5.3$
		NADPH	$13.3 \pm 1.4$	$12.8 \pm 1.6$
	Xylitol dehydrogenase (XDH)	$\text{NAD}^+$	$86.8 \pm 2.5$	$22.6 \pm 0.7$
		$\text{NADP}^+$	-	-

<sup>a</sup> *S. passalidarum* and *P. stipitis* were anaerobically incubated overnight in HD medium with 30 g/L glucose and 30 g/L xylose

<sup>b</sup> For XR,  $K_{m1}$  is the Michaelis constants for NAD(P)H  
For XDH,  $K_{m1}$  is the Michaelis constants for  $\text{NAD(P)}^+$

<sup>c</sup> For XR,  $K_{m2}$  is the Michaelis constants for xylose  
For XDH,  $K_{m2}$  is the Michaelis constants for xylitol

- Not determined due to the undetectable enzymatic activity

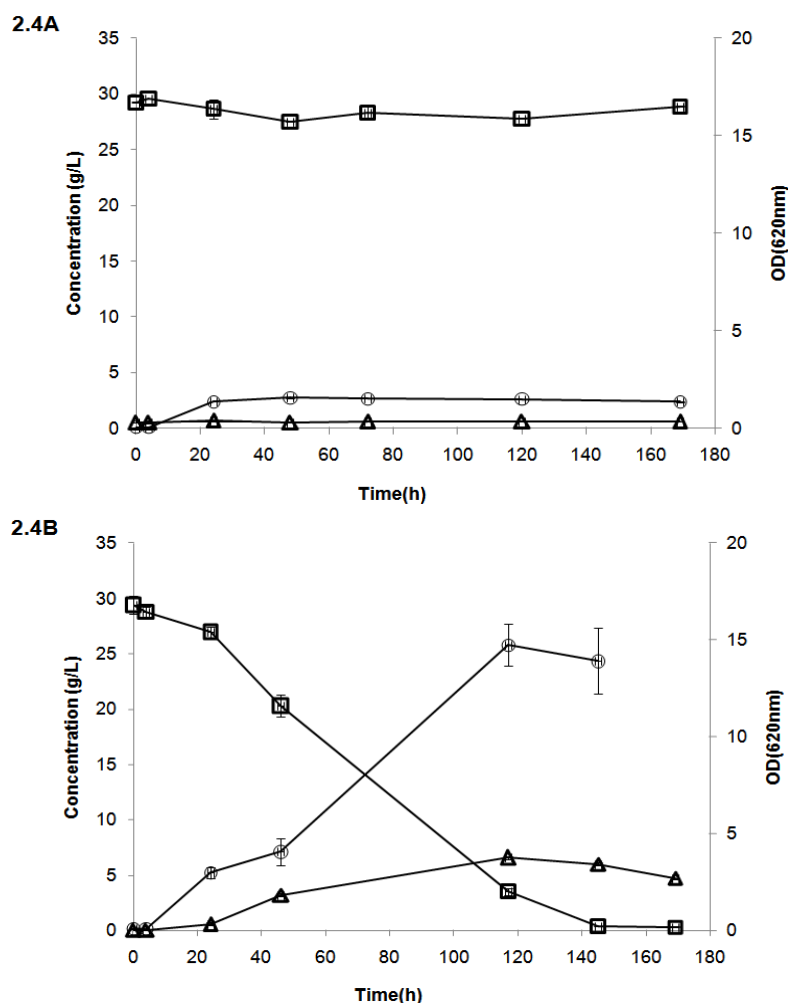
### ***Glucose repression***

Under anaerobic conditions *S. passalidarum* first consumed glucose and then xylose (Figure 2.3A) while glucose and xylose were consumed simultaneously under aerobic conditions (Figure 2.3C). By substituting glucose with the non-metabolised glucose analogue, 2-deoxy-D-glucose (2-DG), xylose consumption and cell growth of *S. passalidarum* under anaerobic conditions was completely inhibited (Figure 2.4A), while xylose consumption was partially inhibited under aerobic conditions, as in the form of an extended lag-phase (Figure 2.4B). Decreased cell growth rate and sugar consumption rate were obvious with the presence of 2-DG under aerobic conditions (comparing Figure 2.4B and Figure 2.3C). These results indicate that xylose uptake or utilization or both were repressed by glucose, in *S. passalidarum*, to different extensions under anaerobic and aerobic conditions.

In order to gain further insight into the influence of glucose on the capacity of *S. passalidarum* to utilize xylose, the enzymatic activities of XR and XDH were determined. Activities were the highest when cells were grown in a medium with only xylose as carbon source, intermediate in cells grown in a medium with equal amounts of glucose and xylose, and the lowest in cells grown in a medium with only glucose (Table 2.4). Adding glucose to the medium that originally contained only xylose resulted in a reduction of the activities of XR and XDH after 6 hours of growth (Table 2.4). The similar trend was found in another xylose fermenting yeast *P. stipitis*, with the only exception of the un-repressed enzymatic activity of XDH in *P. stipitis* at 6 hours after glucose had been added to HDX medium. It might be due to the induction by previously accumulated xylitol in *P. stipitis* and 6 hours (i.e. around 3 to 4 doubling time for *P. stipitis*) is not long enough for cells to sense the substrate change.

With 100% as arbitrary values of the enzymatic activities of XR and XDH from cells incubated in the medium with only xylose, the corresponding values for *S. passalidarum* grown in the medium

with only glucose were 48% and 9%, respectively (Table 2.4). In comparison, the enzymatic activities of *P. stipitis* XR and *P. stipitis* XDH were reduced to around 8% and 6%, respectively (Table 2.4). Thus, the *in vitro* measurement of enzymatic activities of XR and XDH suggest that glucose repressed the activities of these enzymes from both *S. passalidarum* and *P. stipitis*, but the glucose repression on *S. passalidarum* XR/XDH was less pronounced than that observed on *P. stipitis* XR/XDH.



**Figure 2.4** Cell growth, sugar consumption and ethanol production of *S. passalidarum* in HD medium with addition of 0.1% 2-deoxy-D-glucose and 30 g/L xylose under anaerobic (2.4A) and aerobic (2.4B) conditions; xylose (empty square), ethanol (empty triangle), biomass OD (open circle). Data presented are average of two independent experiments and error bars indicate the ranges of measurements.

**Table 2.4** *In vitro* measurement of enzymatic activities of XR and XDH from *S. passalidarum* and *P. stipitis* grown anaerobically in HD medium with different sugars

Strain	<i>S. passalidarum</i>				<i>P. stipitis</i>			
Enzyme	XR		XDH		XR		XDH	
Cofator	NADPH	NADH	NADP <sup>+</sup>	NAD <sup>+</sup>	NADPH	NADH	NADP <sup>+</sup>	NAD <sup>+</sup>
Medium and condition	Specific activity (Unit / mg protein) <sup>d</sup> Relative activity (%)							
<b>HDX medium</b> <sup>a</sup> 16 hours anaerobic incubation	0.24	0.86	N.D.	0.44	0.73	0.65	N.D.	0.31
	100	100	-	100	100	100	-	100
<b>HDX+D medium</b> 16 hours anaerobic incubation in HDX medium and 6 hours more anaerobic incubation after the addition of 30 g/L glucose	0.22	0.55	N.D.	0.20	0.63	0.53	N.D.	0.32
	92	64	-	45	86	82	-	100
<b>HDDX medium</b> <sup>b</sup> 16 hours anaerobic incubation	0.11	0.51	N.D.	0.16	0.26	0.15	N.D.	0.04
	46	59	-	36	36	23	-	13
<b>HDD medium</b> <sup>c</sup> 16 hours anaerobic incubation	0.06	0.41	N.D.	0.04	0.06	0.06	N.D.	0.02
	25	48	-	9	8	9	-	6

<sup>a</sup>. HDX medium: HD medium with 30 g/L xylose

<sup>b</sup>. HDDX medium: HD medium with 30 g/L glucose and 30 g/L xylose

<sup>c</sup>. HDD medium: HD medium with 30 g/L glucose

<sup>d</sup>. One Unit is defined as the amount of enzyme catalyzing the oxidation or reduction of 1  $\mu$ mol of cofactor per minute under the assay conditions. Data presented are average of duplicate experiments.

N.D. Not Detected

-. Not determined

## 2.5 Discussion

Xylose fermentation by yeast such as *P. stipitis* (Agbogbo and Coward-Kelly 2008; Skoog and Hahn-Hägerdal 1990) or by the recombinant *Saccharomyces* yeast with functional expressed XR and XDH (Karhumaa et al. 2007) showed that microaerophilic conditions accurately controlled by oxygen flow is required in order to keep the redox balance of XR-XDH pathway. Recombinant



baker's yeast strains with expression of heterologous filamentous fungi xylose isomerase (XI) do not require the trace amount of oxygen since xylose isomerase can catalyze the direct isomerisation of xylose to xylulose without cofactor requirement (van Maris et al. 2007; Kuyper et al. 2005). However, the recombinant *S. cerevisiae* strain expressing XI showed a significantly lower xylose consumption rate and lower specific ethanol productivity than the recombinant *S. cerevisiae* expressing XR and XDH (Karhumaa et al. 2007), indicating that an efficient XR-XDH pathway could be of importance for the efficient anaerobic xylose fermentation.

In this study, the novel xylose fermenting yeast *S. passalidarum* (Nguyen et al. 2006) showed quick and complete fermentation of xylose with high ethanol yield under anaerobic conditions, while the other traditional xylose fermenting yeast *P. stipitis* almost did not ferment xylose under the same condition. Moreover, *S. passalidarum* displayed an intermediate xylitol yield from consumed xylose (i.e. lower than *P. stipitis* but higher than a XI-expressed *S. cerevisiae*). This could be explained by a more balanced NADH-NAD<sup>+</sup> supply and demand relation between XR and XDH, or an existence of both XR-XDH pathway and XI pathway in *S. passalidarum*. However activity of XI was not detected (using the method described by Belfaquirh and Penninckx (2000), data not show) but a more balanced supply and demand relation of cofactors between XR and XDH was demonstrated. *S. passalidarum* XR has a preference for NADH and *S. passalidarum* XDH is NAD<sup>+</sup> dependent. Therefore, the NAD<sup>+</sup> demanded by XDH can be satisfied by the re-oxidation of NADH by XR in *S. passalidarum*, with no need of extra oxygen. As a control, *P. stipitis* XR showed NADPH preference and *P. stipitis* XDH showed NAD<sup>+</sup> dependence in this study. Only one other XR from *Candida parapsilosis* (CpXR; accession no. AY193716) has been reported previously to have a preference for NADH (Lee et al. 2003). Changing a NADPH-preferred XR towards NADH-preference by genetic engineering has been accomplished in several labs, and the positive effect of NADH-preferred XR on ethanol yield and ethanol productivity has been

demonstrated (Bengtsson et al. 2009; Petschacher and Nidetzky 2008; Jeppsson et al. 2006). The discovery of the NADH-preference of *S. passalidarum* XR enlarges the pool of the natural NADH-preferred XRs. Further purifying, sequencing and studying *S. passalidarum* XR provide a possibility for clarifying the mechanism of the cofactor preference of XR. Such information can be useful in the rational designing of cofactor modification of other XRs, or can provide an easier solution (i.e. without requiring the alteration of coenzyme preference) to the problems caused by cofactor imbalance.

*S. passalidarum* exhibits under anaerobic conditions sequential glucose and xylose consumption (i.e. xylose consumption started after glucose depletion) and under aerobic conditions simultaneous glucose and xylose consumption. This suggests that *S. passalidarum* may use different xylose transport systems under anaerobic and aerobic conditions. There are two xylose transport systems in yeasts: one is the low-affinity facilitated diffusion driven only by the sugar gradient, and the other is the high-affinity xylose-proton symport driven by the proton motive force with the consumption of ATP (Figure 1.3, Section 1.3.2.1.2; Hahn-Hägerdal et al. 2007). In *S. cerevisiae*, xylose is transported only by the hexose transport proteins for facilitated diffusion. The affinity of the hexose transport protein for xylose is one to two orders of magnitude lower than that for glucose. Therefore, during glucose and xylose fermentation by a recombinant xylose-fermenting *S. cerevisiae*, xylose can only be transported and metabolized after glucose has been depleted (Kötter and Ciriacy 1993). In xylose fermenting yeasts such as *Candida intermedia*, xylose can be transported either by the low-affinity facilitated diffusion like in *S. cerevisiae* or by a high-affinity xylose-proton symport, depending on the growth conditions of the yeast (Leandro et al. 2006). Active transport (xylose-proton symport) causes the depletion of conserved ATP under anaerobic conditions and therefore is not quite likely to be used by yeast under anaerobic conditions. It is not uncommon to find only one xylose transport process functioning under some growth conditions

while both transport processes operating in parallel under some other growth conditions (SpencerMartins 1994). For example *Kluyveromyces marxianus*, a naturally xylose utilizing yeast, showed xylose transport activity of only low-affinity facilitated diffusion under microaerobic conditions while activities of both low-affinity facilitated diffusion and high-affinity active transport under aerobic conditions. These different transport systems allowed *K. marxianus* to sequentially utilize glucose and xylose under microaerobic conditions while simultaneously utilize glucose and xylose under aerobic conditions (Stambuk et al. 2003). Therefore, it is reasonable to speculate that *S. passalidarum*, similar to *K. marxianus*, has the similar tends of utilizing two different xylose transport systems under anaerobic and aerobic conditions. Moreover, the rapid growth rate of *S. passalidarum* on xylose under anaerobic conditions might be due to the high capacity of the low-affinity facilitated xylose transporters, as being discovered in the rapid growing *K. marxianus* under the microaerobic condition (Stambuk et al. 2003).

Substituting glucose with a non-metabolizable glucose analogue, 2-deoxy-D-glucose (2-DG) further supported the above notion. 2-DG has a similar affinity as glucose to hexose transporters but can not be metabolized by cells (Pelicano et al. 2006). With the presence of 2-DG, the xylose consumption of *S. passalidarum* was completely inhibited under anaerobic conditions, while xylose can still be consumed though a decreased xylose consumption rate was observed under aerobic conditions. This decreased xylose consumption rate under aerobic conditions can be explained by the decreased capacity of xylose transport caused by 2-DG, possibly due to the two reasons: first, the low-affinity facilitated diffusion process was blocked as transporters were occupied by 2-DG; second, the amount of cellular ATP required for active transport was decreased because of the ATP consumption caused by the phosphorylation of 2-DG into non-metabolizable 2-DG-P, catalyzed by hexokinase (Pelicano et al. 2006).

Moreover, the complete inhibition of xylose consumption by 2-DG under anaerobic conditions might also be due to the lethal effect of cellular ATP depletion caused by 2-DG. However, different yeast strains have different levels of resistance to 2-DG, and a concentration of 0.1% as applied in this study is the dosage that can be tolerated by many yeast strains (Bala et al. 2001; Herve et al. 1992; Heredia and Heredia 1988). Since *S. passalidarum* was not growth inhibited and glucose was completely consumed in a medium containing 0.1% 2-DG and 3% glucose under anaerobic conditions (data not shown), the possibility of lethal effect caused by 0.1% 2-DG can be excluded.

The presence of glucose also caused a significant repression of the enzymatic activity of XR and XDH, in both *P. stipitis* and *S. passalidarum*. It is known that the activity of XR and XDH can be induced by xylose and xylitol, respectively, while glucose can repress this induction. Glucose repressions with varying degrees on the induction of XR and XDH activity have been reported. For example, glucose partially repressed the induction of XR and XDH in *P. stipitis* (Bicho et al. 1988) but strongly repress the aldose reductase activity in *Candida guilliermondii* (Lee et al. 2003). In this study, we also found that the glucose repressions of XR and XDH in *S. passalidarum* were less pronounced than the repression of XR and XDH in *P. stipitis*.

In conclusion, *S. passalidarum* did excellent xylose fermentation under anaerobic conditions, which could be explained by the cofactor balance between its NADH-preferred XR and NAD<sup>+</sup>-dependent XDH. The presence of glucose may affect the xylose metabolism in *S. passalidarum* in two ways. Firstly, glucose may block the facilitated diffusion of xylose, which probably is the only way of xylose transport under anaerobic conditions, by occupying the transporters. Secondly, the presence of intracellular glucose may cause the signal repression of the xylose induced synthesis of XR and XDH (Webb and Lee 1991). More detailed studies on the mechanisms of the effects of glucose on xylose uptake by *S. passalidarum* will be useful to further improve the fermentation

efficiency of this strain, in medium with a mixture of glucose and xylose as present in the lignocellulosic hydrolysate for industrial bioethanol production.

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# Chapter 3

## Effects of furfural and 5-hydroxymethylfurfural on the anaerobic fermentation by *Spathaspora passalidarum*

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### 3.1 Abstract

The inhibitory effects of furfural and 5-hydroxymethylfurfural (HMF) on the yeast *Spathaspora passalidarum* characterized by an excellent anaerobic xylose fermentation performance was studied in order to estimate the potential of this yeast for application in industrial lignocellulosic ethanol production. Furfural and HMF are two commonly encountered inhibitors present in pretreated lignocellulosic biomass. With *S. passalidarum* furfural was a stronger inhibitor than HMF. Furfural was converted to furfuryl alcohol and HMF was also degraded by *S. passalidarum*. Nevertheless, even though furfural and HMF had completely disappeared, inhibition on cell growth, sugar consumption and ethanol production was still pronounced. This is unlike *S. cerevisiae* that resumed growth and fermentation capacity upon complete conversion of furfural and HMF. With *S. passalidarum*, increasing the concentration of furfural or HMF led to the reduction of cell biomass, sugar consumption, glycerol yield and ethanol yield during the anaerobic fermentation. The enzymes involved in both furfural and HMF reductions showed cofactor preference for NADH. The presence of xylose in the medium with furfural or HMF slightly improved the fermentation performance of *S. passalidarum*, which is probably due to the xylose induction of responsible enzymes. Possible inhibition mechanisms of furfural and HMF on *S. passalidarum* are discussed.

### 3.2 Introduction

It has become important to develop alternative energy sources to reduce environmental pollution, eliminate global warming and secure an energy supply. Bioethanol production from potential low-cost lignocellulosic biomass is one of these efforts. Based on today's technology, the lignocellulosic bioethanol production is mainly composed of three steps: (1) pretreatment to loosen the compact cell wall, solubilize the hemicellulose and make cellulose more accessible to enzyme attack; (2) addition of hydrolytic enzymes, e.g. cellulase and hemicellulase, to release sugars like glucose from cellulose and xylose from hemicellulose; and (3) fermentation to convert the released sugars into ethanol (van Maris AJ et al. 2006). The most commonly applied fermentation microorganism is baker's yeast, *S. cerevisiae*. This yeast can only ferment glucose into ethanol, leaving large amounts of xylose unfermented. Most xylose-fermenting yeasts such as *Pichia* and *Candida* species can only ferment xylose into ethanol under strictly controlled microaerophilic conditions which is very costly (Skoog and Hahn-Hägerdal 1990). In Chapter 2, a novel xylose fermenting yeast, *S. passalidarum*, was shown to produce ethanol from glucose and xylose efficiently under anaerobic conditions. This property makes this yeast a promising candidate for the cost-effective lignocellulosic ethanol production. However, before applying this yeast into industrial lignocellulosic ethanol production process, its tolerance to inhibitors present in the pretreated lignocellulosic hydrolysate needs to be elucidated.

Due to the harsh conditions applied by the presently available pretreatment methods, e.g. high temperature/pressure and addition of acids/oxidants, a wide range of inhibitors are generated after pretreatment of lignocellulosic biomass. These inhibitors may cause the severe reduction of ethanol yield and ethanol productivity by affecting the fermentation performance of the microorganisms (Klinke et al. 2004). Generally speaking, pretreatment-generated inhibitors can be categorized into three groups: furans, weak acids and phenolic compounds. Furans are generated as result of the

dehydration of free sugars, e.g. furfural from xylose and 5-hydroxymethylfurfuryl (HMF) from glucose. Weak acids are also formed, e.g. acetic acid from deacetylation of hemicelluloses and formic acid as well as levulinic acid from break-down of furans. Furthermore, phenol compounds are generated by the breakdown of lignin (Palmqvist and Hahn-Hägerdal 2000b). The mechanisms by which these compounds inhibit fermentation are still not completely clear (Almeida et al. 2007). Previous studies have shown furfural as a key contributor to the lignocellulosic hydrolysate toxicity for *S. cerevisiae* (Heer and Sauer 2008) and furfural levels directly correlate with toxicity (Martínez et al. 2001). HMF, chemically related with furfural, is another commonly encountered pretreatment-generated inhibitor. The harmful effects of furfural and HMF to *S. cerevisiae* have been attributed to their interference with yeast metabolism by inhibiting key enzymes in the central carbon metabolism or by reducing intracellular ATP and NAD(P)H levels (Palmqvist and Hahn-Hägerdal 2000b).

In order to estimate the potential of applying the novel xylose fermenting yeast *S. passalidarum* in industrial lignocellulosic bioethanol production, the inhibition effects of furfural and HMF on the anaerobic fermentation of this yeast was investigated.

### **3.3 Materials and Methods**

#### ***Media***

The culture and the selection media used in this study are listed below: (1) Yeast extract peptone dextrose (YPD) medium: 10 g/L yeast extract, 20 g/L peptone, and 30 g/L glucose; (2) Yeast extract peptone xylose (YPX) medium: 10 g/L yeast extract, 20 g/L peptone, and 30 g/L xylose; (3) Yeast extract peptone dextrose and xylose (YPDX) medium contains 10 g/L yeast extract, 20 g/L peptone, 30 g/L glucose and 30 g/L xylose.

Different concentration of furfural or HMF was added into the medium at the start of the inhibition fermentation as indicated.

### ***Yeast cultivations***

Yeast strains *S. cerevisiae* ATCC 96581 and *Spathaspora passalidarum* ATCC MYA-4345 were used in this study. Both of the yeast strains were from the American Type Culture Collection (ATCC). *S. cerevisiae* ATCC 96581 is a heavily flocculating baker's yeast isolated from a spent sulphite liquor fermentation plant (Linden et al. 1992). *S. passalidarum* is a xylose-fermenting yeast associated with wood-boring beetles (Nguyen et al. 2006). The propagation and pre-culture preparation of the strains were according to the procedure described in Chapter 2.

### ***Fermentation***

All anaerobic fermentations were performed as batch fermentation using 50 ml volume in 100 ml anaerobic vials. In order to support initial cell biomass growth, residual oxygen in the medium and head space of the vial was not flushed out. The vials were inoculated with a pre-culture at OD<sub>620nm</sub> of circa 0.05. Different concentrations of furfural or HMF were added at the start of the fermentation, as indicated. The vials were incubated at 32°C in an orbital shaker with rotation speed of 120 rpm.

Samples were taken under sterile conditions. Cell growth was monitored during the fermentation by measuring the optical density at 620 nm using Ultrospec 3000 (Pharmacia Biotech, Sweden). After fermentation, the dry weight of the biomass was estimated by filtering through a membrane filter (0.45 µm) and drying the filter overnight at 105°C. The carbon recovery was calculated as described in Chapter 2.

### ***Chemical analysis***

Glucose, xylose, ethanol, xylitol, glycerol and acetic acid were analyzed by high performance liquid chromatography (HPLC) system (Shimadzu, Japan) using refractive index (RI) detector equipped with an Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) running at 63°C with 4 mM H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.6 ml/min.

HMF and furfural were analyzed by the same HPLC system using the UV detection at 250 nm in a SPD-M10AVP diode array detector equipped with an Aminex HPX-87H column running at 63°C with 4 mM H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.6 ml/min.

Furfuryl alcohol was analyzed by the same HPLC system using the UV detection at 210 nm in a SPD-M20AVP diode array detector equipped with an Aminex HPX-87P column running at 80°C with H<sub>2</sub>O as eluent at a flow rate of 0.5 ml/min.

### ***Furfural, HMF and xylose reduction activity assay***

For *in vitro* measurements of furfural, HMF and xylose reduction activity, yeast cells were grown overnight in YPD, YPX or YPDX medium as indicated. Cells were harvested and disrupted as described in Chapter 2. The final homogenates were centrifuged at 14000 rpm for 5 minutes at 2°C and supernatants were used for enzymatic activity measurement. Protein concentrations in the cell-free extract were determined by “Quick Start™ Bradford Protein Assay Kit” (Bio-Rad Laboratories Ltd., USA) according to the manufacturer’s instruction.

*In vitro* furfural, HMF and xylose reduction activities were determined spectrophotometrically by following the oxidation of NAD(P)H at 340 nm. Dynamic measurement of the absorbance at 340 nm ( $A_{340\text{nm}}$ ) was carried out using a Lambda 25 UV/VIS spectrometer (PerkinElmer, USA) at 25°C, with interval time of 5 seconds for recording and a total measuring time of 5 minutes for each

reaction. Cell-free extracts were diluted, if necessary, to ensure the constancy of  $\Delta(A_{340\text{nm}})/\Delta(\text{time})$  lasting for at least 1 minute.

Furfural reduction activity was measured in the reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0); 0.4 mM NAD(P)H; 10 mM furfural; cell-free extracts; and distilled water in a total volume of 400  $\mu\text{l}$ . HMF reduction activity was measured using the same reaction mixture as described above except for replacing 10 mM furfural with 10 mM HMF. Xylose reduction activity was measured using the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0); 0.4 mM NAD(P)H; 50 mM D-xylose; cell-free extract; and distilled water in a total volume of 400  $\mu\text{l}$ .

### 3.4 Results

#### ***Inhibition effects of furfural and HMF on the anaerobic glucose fermentation by S. passalidarum***

The effects of various concentrations of furfural and HMF (0 - 3 g/L) on the anaerobic glucose fermentation by *S. passalidarum* were studied. With increasing concentrations of furfural or HMF, cell growth decreased (Figure 3.1), sugar consumption and final ethanol concentration decreased dramatically, and ethanol yield decreased slightly (Table 3.1). The anaerobic cell growth of *S. passalidarum* was more inhibited by furfural than by HMF. For example, 0.5 g/L furfural caused a 40~50% of inhibition on cell growth of *S. passalidarum*, while 1~2 g/L HMF was required for a similar effect (Figure 3.1). The glycerol yield, as determined when furfural or HMF was completely converted, declined from 0.02 g/g-consumed-glucose to almost 0 when the concentration of furfural or HMF increased from 0.25 to 3 g/L (Table 3.1). Glycerol formation was absent during the time when the furfural or HMF concentration in the medium was above approximately 0.5 g/L or 1.5 g/L, respectively (data not shown). Glycerol, however, was formed along with the furfural or HMF

reduction when the furfural or HMF concentration was below 0.5 g/L or 1.5 g/L, respectively (Figure 3.2 and Figure 3.3). The acetic acid yield increased from 0.002 g/g consumed glucose to 0.029 g/g consumed glucose when furfural concentration increased from 0 to 0.5 g/L, while it decreased to a stable value of around 0.01 g/g consumed glucose when the furfural concentration further increased from 0.5 g/L to 2 g/L. In comparison, the acetic acid yield increased from 0.002 g/g consumed glucose to 0.014 g/g consumed glucose along with the addition of HMF concentration rising from 0 to 3 g/L (Table 3.1).

Representative glucose fermentation profiles with *S. passalidarum* with addition of 0.5 g/L furfural or 1 g/L HMF are presented in Figure 3.2 and Figure 3.3. Furfural at an initial concentration of 0.5 g/L was 95 % converted to furfuryl alcohol within 48 hours (Figure 3.2), but the sugar consumption and cell growth of *S. passalidarum* remained inhibited until 150 hours. A similar pattern was observed with the addition of 1 g/L HMF (Figure 3.3). *S. cerevisiae* and *P. stipitis* are able to reduce HMF to 2,5-bis-hydroxymethylfuran (Liu and Moon 2009) and it is possible that this is also the case for *S. passalidarum*, but 2,5-bis-hydroxymethylfuran was not analyzed in this study since the compound is not commercially available.

The inhibition effects of furfuryl alcohol, the conversion product of furfural, on *S. passalidarum* were also examined. Furfuryl alcohol is a much less toxic compound than furfural (Table 3.3). Only a slight reduction of cell specific growth rate and glucose consumption rate were observed, even when furfuryl alcohol concentration reached to as high as 2 to 3 g/L. Ethanol yield was not affected by the presence of furfuryl alcohol.

**Table 3.1** Inhibition effects of furfural (left column) and HMF (right column) on the anaerobic glucose fermentation of *S. passalidarum*

Inhibitor concentration (g/L)		Glucose consumption (%)		Final ethanol concentration (g/L)		Ethanol yield (g/g-consumed-glucose)		Acetic acid yield (g/g-consumed-glucose)		Glycerol yield (g/g-consumed-glucose)		Carbon recovery (%)	
Furfural	HMF												
0	0	100 <sup>a</sup>	100 <sup>a</sup>	13.1 <sup>a</sup>	13.1 <sup>a</sup>	0.44 <sup>a</sup>	0.44 <sup>a</sup>	0.002 <sup>a</sup>	0.002 <sup>a</sup>	0.018 <sup>a</sup>	0.018 <sup>a</sup>	95.7 <sup>a</sup>	95.7 <sup>a</sup>
0.25	0.25	50.4 <sup>c</sup>	81.9 <sup>c</sup>	5.6 <sup>c</sup>	10.3 <sup>c</sup>	0.36 <sup>c</sup>	0.39 <sup>c</sup>	0.021 <sup>c</sup>	0.002 <sup>c</sup>	0.019 <sup>b</sup>	0.015 <sup>b</sup>	90.2 <sup>c</sup>	91.8 <sup>c</sup>
0.5	0.5	41.3 <sup>c</sup>	75.1 <sup>c</sup>	5.2 <sup>c</sup>	8.6 <sup>c</sup>	0.38 <sup>c</sup>	0.38 <sup>c</sup>	0.029 <sup>c</sup>	0.004 <sup>c</sup>	0.007 <sup>b</sup>	0.012 <sup>b</sup>	93.9 <sup>c</sup>	90.2 <sup>c</sup>
1	1	29.3 <sup>c</sup>	61.9 <sup>c</sup>	3.5 <sup>c</sup>	7.1 <sup>c</sup>	0.37 <sup>c</sup>	0.42 <sup>c</sup>	0.010 <sup>c</sup>	0.005 <sup>c</sup>	0.004 <sup>b</sup>	0.011 <sup>b</sup>	89.5 <sup>c</sup>	97.8 <sup>c</sup>
2	2	23.3 <sup>c</sup>	39.1 <sup>c</sup>	3.0 <sup>c</sup>	3.8 <sup>c</sup>	0.42 <sup>c</sup>	0.37 <sup>c</sup>	0.011 <sup>c</sup>	0.007 <sup>c</sup>	0.002 <sup>b</sup>	0.009 <sup>b</sup>	98.2 <sup>c</sup>	108.9 <sup>c</sup>
3	3	0 <sup>c</sup>	27.2 <sup>c</sup>	-	2.9 <sup>c</sup>	-	0.37 <sup>c</sup>	-	0.014 <sup>c</sup>	-	0.005 <sup>b</sup>	-	90.6 <sup>c</sup>

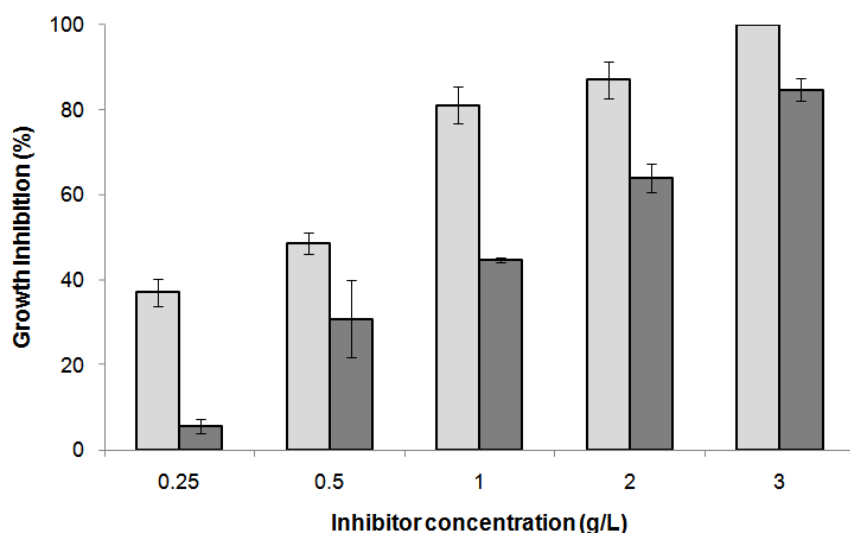
<sup>a</sup> Values were calculated after 72 hours of anaerobic fermentation.

<sup>b</sup> Glycerol yields in the inhibition media were calculated at the time when furfural or HMF was completely converted.

<sup>c</sup> Values were calculated after 150 hours of anaerobic fermentation.

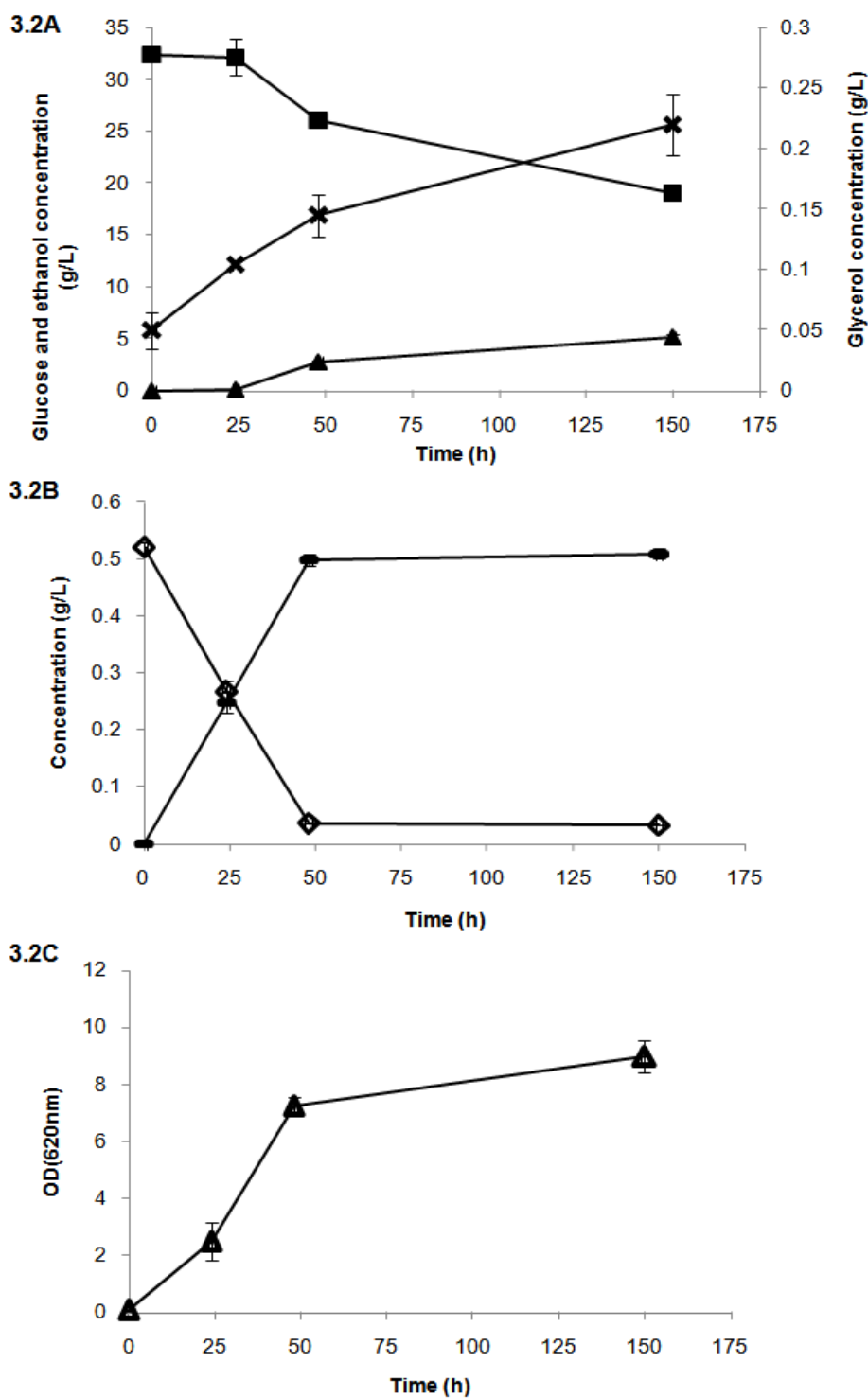
-, Not detected.

Data presented are average of duplicate experiments.

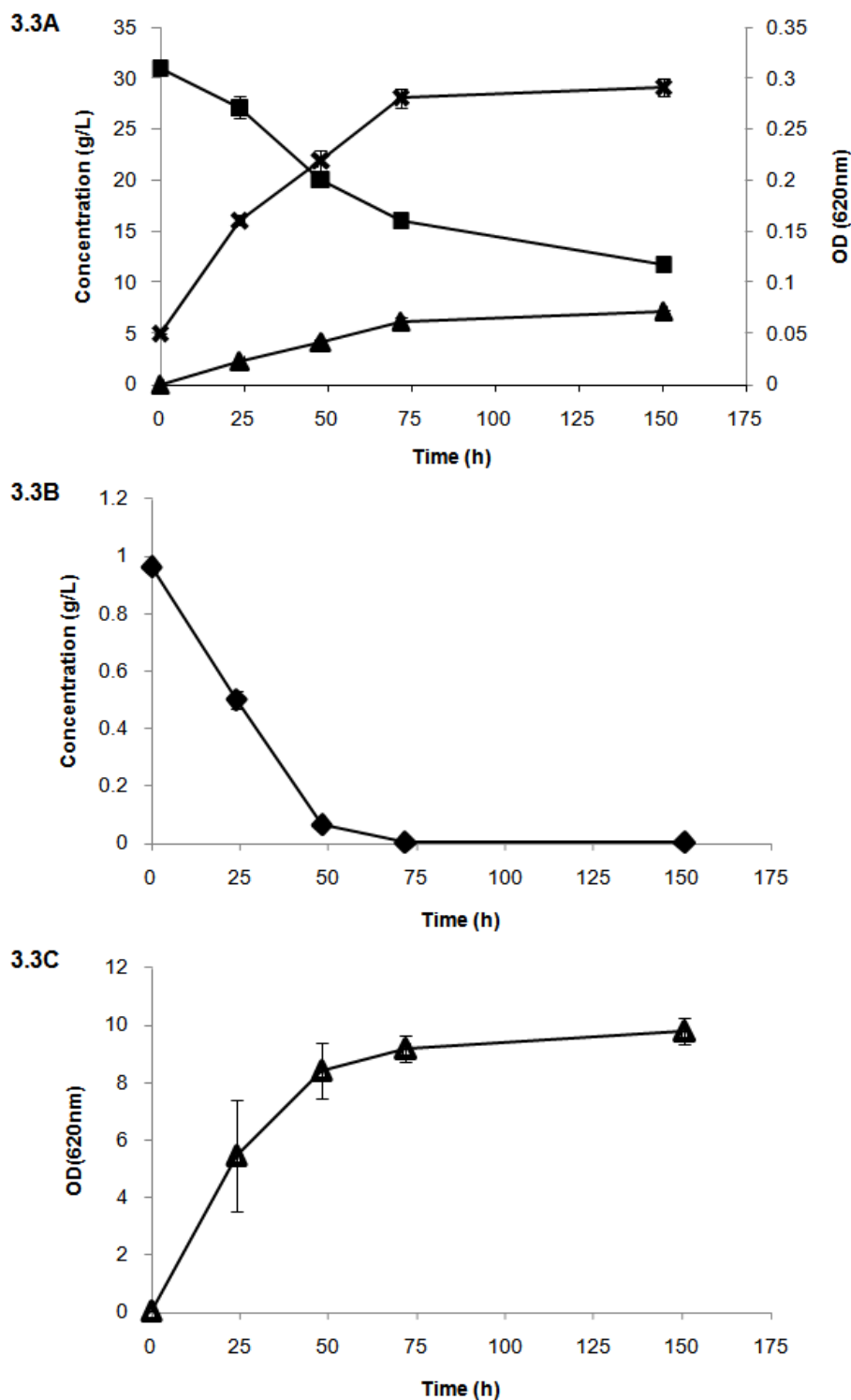


**Figure 3.1** Cell growth inhibition of *S. passalidarum* in YPD medium by different concentrations of furfural or HMF (0.25, 0.5, 1, 2, 3 g/L); furfural (light column); HMF (dark column). Growth inhibition was calculated at 48h as following: Growth inhibition (%) =  $[1 - \text{OD}_{620\text{nm}} (\text{medium with } x \text{ g/L inhibitor}) / \text{OD}_{620\text{nm}} (\text{medium without inhibitor})] \times 100\%$ . Cells were grown in 100 ml anaerobic vials with 50 ml medium. Vials were incubated at 32 °C in an orbital shaker with rotation speed of 120 rpm. Data presented are average of triplicate experiments and error bars indicate the standard deviations.





**Figure 3.2** Anaerobic fermentation of *S. passalidarum* in YPD medium with 0.5 g/L furfural. **3.2A:** Glucose consumption, ethanol production and glycerol formation; glucose (*filled square*); ethanol (*filled triangle*); glycerol (×); **3.2B:** Furfural reduction and furfuryl alcohol production; furfural (*open diamond*); furfuryl alcohol (-) **3.2C:** Biomass growth. The final OD<sub>620nm</sub> of *S. passalidarum* in YPD medium (control) is  $17.7 \pm 1.6$ . Data presented are average of duplicate experiments. Error bars indicate the ranges of measurements.



**Figure 3.3** Anaerobic fermentation of *S. passalidarum* in YPD medium with 1 g/L HMF. **3.3A:** Glucose consumption, ethanol production and glycerol formation; glucose (*filled square*); ethanol (*filled triangle*); glycerol (×); **3.3B:** HMF reduction; **3.3C:** Biomass growth. The final OD<sub>620nm</sub> of *S. passalidarum* in YPD medium (control) is  $17.7 \pm 1.6$ . Data presented are average of duplicate experiments. Error bars indicate the ranges of measurements.

**Table 3.2** Anaerobic glucose fermentation of *S. passalidarum* in the presence of increasing amounts of furfuryl alcohol

Furfuryl alcohol concentration (g/L)	Specific growth rate (%) <sup>a</sup>	Glucose consumption after 32 hours (%)	Ethanol concentration after 32 hours	Ethanol yield (g/g-consumed-glucose)	Acetic acid yield (g/g-consumed-glucose)	Glycerol yield (g/g-consumed-glucose)	Carbon recovery (%)
0	100	100	13.0	0.42	0.003	0.018	96.6
0.5	95	100	13.3	0.43	0.004	0.021	99.8
1	95	100	13.0	0.42	0.009	0.019	95.7
2	92	75.2	10.7	0.46	0.014	0.021	103.7
3	82	54.2	6.9	0.41	0.025	0.014	90.9

<sup>a</sup> The specific growth rates were calculated by the slope of the straight line from the plot of LnOD<sub>620nm</sub> versus time. 100% was equivalent to 0.67 h<sup>-1</sup>.

Data presented are average of duplicate experiments.

### *Slightly increased inhibitor tolerance of S. passalidarum during the anaerobic xylose fermentation*

It was observed that the aerobic growth of *S. passalidarum* was inhibited by furfural/HMF, and therefore halos around filterpaper discs containing furfural/HMF were formed on agar plates. The diameters of the halos on agar plate with xylose were smaller than those on agar plate with glucose (not shown). These observations indicate that furfural or HMF was less toxic to *S. passalidarum* with xylose than with glucose under aerobic conditions. The less toxic effect of furfural or HMF to *S. passalidarum* in the medium with xylose was also observed under anaerobic conditions (Table 3.3). The percentage biomass growth and the sugar consumption of *S. passalidarum* in xylose medium with 1 g/L furfural or HMF were higher than those observed in glucose medium. Moreover, the average *in vivo* inhibitor reduction rate of furfural and HMF were also slightly improved with the presence of xylose (Table 3.3).

**Table 3.3** Anaerobic glucose or xylose fermentation by *S. passalidarum* in the presence of furfural or HMF <sup>a</sup>

Inhibitor	Sugar	Percentage biomass growth (%)	Sugar consumption (%)	Final ethanol concentration (g/L)	Ethanol yield (g/g-consumed-sugar)	Glycerol yield (g/g-consumed-sugar)	Acetic acid yield (g/g-consumed-sugar)	Inhibitor left after 32 hours (g/L)	Average inhibitor reduction rate in 32 hours (gL <sup>-1</sup> h <sup>-1</sup> )
1 g/L Furfural	Glucose	20.5 <sup>b</sup>	29.1	4.0	0.39	0.011 <sup>c</sup>	0.012 <sup>d</sup>	0.36	0.022 <sup>e</sup>
	Xylose	38.8 <sup>b</sup>	38.5	4.9	0.39	0.006 <sup>c</sup>	0.008 <sup>d</sup>	0.16	0.027 <sup>e</sup>
1 g/L HMF	Glucose	38.9 <sup>b</sup>	53.9	7.2	0.43	0.013 <sup>c</sup>	0.007 <sup>d</sup>	0.53	0.014 <sup>e</sup>
	Xylose	57.6 <sup>b</sup>	61.1	7.8	0.42	0.007 <sup>c</sup>	0.005 <sup>d</sup>	0.48	0.016 <sup>e</sup>

<sup>a</sup>. Anaerobic fermentations were conducted in YPD or YPX medium with addition of 1 g/L furfural or 1 g/L HMF as indicated. Values were calculated after 120 hours of fermentation, if not specified.

<sup>b</sup>. Cell growths were monitored by measuring the optical density under 620nm and 100% were equivalent to 18.5 in YPD medium and 8.5 in YPX medium.

<sup>c</sup>. The glycerol yield in YPD and YPX medium without inhibitors, as controls, were 0.019 and 0.007, respectively.

<sup>d</sup>. The acetic acid yield in YPD and YPX medium without inhibitors, as controls, were 0.003 and 0.003, respectively.

<sup>e</sup>. All furfural and HMF were converted in 48 hours.

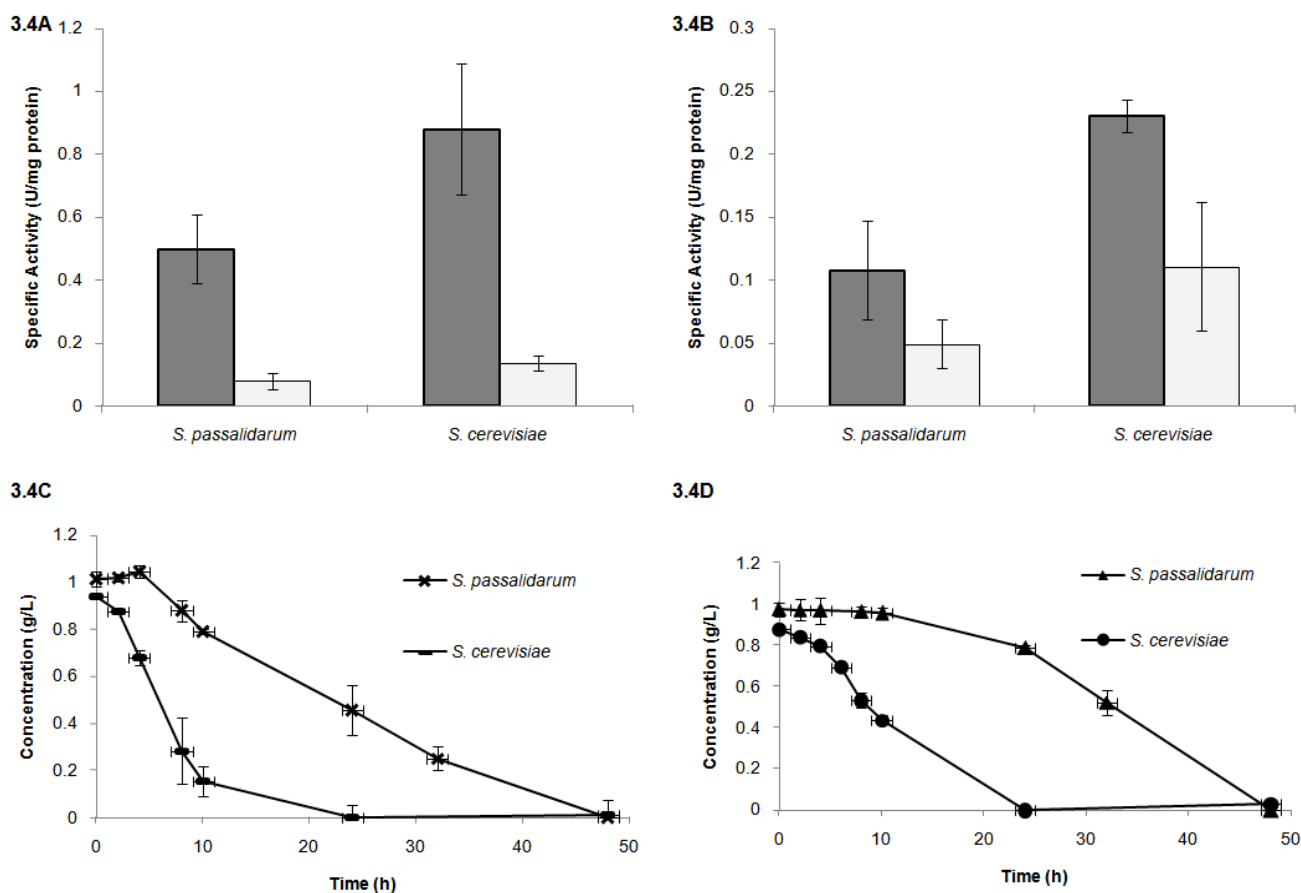
Data presented are average of duplicate experiments.

### ***NADH preference for furfural and HMF reduction by S. passalidarum***

Table 3.4 shows that *S. passalidarum* preferred NADH over NADPH for furfural and HMF reduction. In addition, when *S. passalidarum* were incubated in the medium with xylose (YPX), the *in vitro* furfural reduction activity and HMF reduction activity as well as xylose reduction activity were all increased more than two fold than those from the cells incubated in the medium with glucose (YPD) (Table 3.4). Thus xylose appears to induce these enzymes.

The *in vitro* measurements of furfural and HMF reduction activities and the disappearance of furfural and HMF from the media during the anaerobic fermentation of *S. passalidarum* and *S. cerevisiae* ATCC 96581 were presented (Figure 3.4). *S. cerevisiae* ATCC 96581 is a yeast strain with a high tolerance to pretreatment generated inhibitors (Modig et al. 2008). This strain showed more than two fold higher furfural and HMF reduction activity (Figure 3.4A and 3.4B) than *S. passalidarum*. In addition, 3-5 fold faster disappearance of furfural and HMF from the media

fermented by *S. cerevisiae* ATCC 96581 than from the one fermented by *S. passalidarum* was observed (Figure 3.4C and 3.4D).



**Figure 3.4**

**3.4A:** *In vitro* measurement of furfural reduction activity by *S. passalidarum* or *S. cerevisiae* ATCC 96581 with cofactor of NADH (dark column) or NADPH (light column); **3.4B:** HMF reduction activity by *S. passalidarum* or *S. cerevisiae* ATCC 96581 with cofactor of NADH (dark column) or NADPH (light column); *S. cerevisiae* ATCC 96581 and *S. passalidarum* were incubated anaerobically in YPDX medium for 16 hours before measurements.

**3.4C:** Furfural disappearance in YPDX medium with addition of 1g/L furfural during the anaerobic fermentation by *S. passalidarum* (×) and *S. cerevisiae* ATCC 96581 (-); **3.4D:** HMF disappearance in YPDX medium with addition of 1 g/L HMF during the anaerobic fermentation by *S. passalidarum* (filled triangle) and *S. cerevisiae* ATCC 96581 (filled circle).

Data presented are average of duplicate experiments and error bars indicate the ranges of measurements.

**Table 3.4** Furfural, HMF and xylose reduction activities by *S. passalidarum* <sup>a</sup>

Medium	Reduction activity (Unit/mg protein) <sup>b</sup>					
	Furfural (NADH)	Furfural (NADPH)	HMF (NADH)	HMF (NADPH)	Xylose (NADH)	Xylose (NADPH)
YPD (Glucose)	0.41	0.02	0.05	0.01	0.23	0.07
YPX (Xylose)	0.83	0.05	0.12	0.06	0.56	0.37

<sup>a</sup> *S. passalidarum* were incubated anaerobically in YPD or YPX medium for 16 hours before measurements.

<sup>b</sup> One unit of activity was defined as the amount of enzymes catalyzing the oxidation of 1  $\mu$ mol of NAD(P)H per minute using either furfural, HMF or xylose as substrate.

Data presented are average of two independent measurements.

### 3.5 Discussion

The inhibitory effects of furfural and HMF, two usually abundant inhibitors formed after the pretreatment of lignocellulosic biomass, on the anaerobic fermentation of the novel xylose fermenting yeast *S. passalidarum* were investigated. *S. passalidarum* appears to be much more sensitive to furfural or HMF than *S. cerevisiae*. At 0.5 g/L furfural or 1 g/L HMF, severe inhibitions on the anaerobic fermentation of *S. passalidarum* were observed, resulting in fair amounts of unconsumed sugar and heavily reduced cell growth. In comparison, most *S. cerevisiae* strains tolerate 0.5 g/L furfural or 1 g/L HMF, resulting in normal ethanol yield and ethanol productivity and slightly inhibited cell growth (Almeida et al. 2007; Boyer et al. 1992).

This study demonstrated that *S. passalidarum* is able to reduce furfural to furfuryl alcohol using NADH as cofactor, which is similar to the furfural reduction reaction in *S. cerevisiae* (Villa et al. 1992). HMF reduction in *S. passalidarum* also prefers NADH, which is different from most *S. cerevisiae* strains except for strain ATCC 96581(=TMB3000) which is the only known *S. cerevisiae* strain using NADH as a cofactor for HMF reduction (Nilsson et al. 2005). The inhibition by furfural and HMF of the anaerobic fermentation by *S. passalidarum* include reduced biomass formation, sugar consumption and ethanol production. Furthermore, glycerol yield from consumed sugar was also decreased along with the increasing furfural or HMF concentrations. Under unstressed

conditions, glycerol is reduced from dihydroxyacetone phosphate in order to re-oxidize the surplus NADH derived from primarily anabolic reactions during yeast fermentation (Palmqvist et al. 2000b). Therefore, the reduced glycerol yield along with the increased furfural or HMF concentrations suggests that the reduction of furfural and HMF competes with dihydroxyacetone phosphate for available NADH in *S. passalidarum*.

This study also found that even though furfural and HMF were completely converted, cell growth, sugar consumption and ethanol production by *S. passalidarum* were still severely inhibited for at least 100 hours during anaerobic fermentation. Furfuryl alcohol, the conversion product of furfural, did not inhibit the anaerobic fermentation at concentrations up to 1 g/L. Therefore the unreleased inhibition effects during the anaerobic fermentation of *S. passalidarum* in the medium with 0.5 g/L furfural indicate that other inhibitory compounds derived from furfural metabolism may be generated. The interference of furan on the metabolism of *S. passalidarum* may have the following reasons: (1) furan reductions may lead to NADH imbalance in the cell and therefore disturb cell metabolism; (2) furan reductions may compete or repress some crucial enzymatic reactions that are also involved in furan reduction, causing accumulation of toxic intermediates such as acetaldehyde. As glycerol formation was blocked when the concentration of furan in the medium was high, the role of glycerol may be compromised both as a compatible solute protecting the cell in the high osmolarity environment (Parmar et al. 2011) and as a precursor used to synthesize the cellular membrane (Guo et al. 2011). Furfural has been found to cause membrane damages to vacuoles and mitochondria in yeast such as *S. cerevisiae* (Almeida et al. 2007), so the reduced glycerol formation in *S. passalidarum* may result in impaired membrane repair. Key metabolic enzymes such as alcohol dehydrogenase (ADH) (Laadan et al. 2008; Larroy et al. 2002) and some novel aldehyde reductases (Liu and Moon 2009) are responsible for the reduction of furans by yeasts. Many ADHs, either NADH-dependent or NADPH-dependent, in different yeast strains have been identified and

their ability to catalyze both furan reduction and acetaldehyde reduction to ethanol have been demonstrated (Laadan et al. 2008; Larroy et al. 2002). Substrate competition between furan and acetaldehyde may lead to acetaldehyde accumulation, and acetaldehyde has been suggested to be a cause for the inhibition of cell growth (Jones 1989). A reduced ethanol yield and an increased acetic acid yield from consumed glucose were apparent when the concentration of furfural or HMF increased from 0 to 0.5 g/L (Table 3.1). This increased acetic acid yield may be due to the increasing amounts of acetaldehyde which is due to the competition between furan reduction and acetaldehyde reduction catalyzed by ADH. Acetic acid is converted from acetaldehyde is by catalysis of ALDH. The increasing amount of acetic acid yield along with increasing furfural was much more significant than in with increasing HMF, which agrees well with the more severe inhibiting effects caused by furfural than HMF, as observed in this study. However, when the furfural concentration increased from 0.5 to 1 g/L, the acetic acid yield decreased from 0.03 g/g consumed glucose to around 0.01 g/g consumed glucose and this yield (0.01 g/g consumed glucose) was stable when furfural concentration further increased from 1 g/L to 2 g/L. The possible explanation of this phenomenon could be that besides the substrate competition between furfural and acetaldehyde for the enzyme ADH, furfural also inhibits the enzyme activity of ALDH (Modig et al. 2002). When the furfural concentration increased, such inhibition to ALDH also increased. Therefore, when the furfural concentration further increased, less acetic acid was converted from acetaldehyde, leading to the accumulation of the toxic acetaldehyde.

The lower inhibitor tolerance of *S. passalidarum* compared with that of *S. cerevisiae* may also be due to the lower activities of the enzymes responsible for furfural and HMF reduction, as found in this study. *S. cerevisiae* ATCC 96581 consumed all the glucose and produced ethanol at a yield of around 0.4 g/g consumed glucose after the complete metabolism of furfural or HMF (data not show). *S. cerevisiae* ATCC 96581 has a high capacity for reduction of furan compounds and is the



only *S.cerevisiae* strain with a NADH-preferred HMF reduction mechanism (Nilsson et al. 2005). Although it is hypothesized above that the preference of NADH for furan reduction may cause a NADH imbalance and interfere with basic cell metabolism in *S. passalidarum*, NADH interference may not be that severe in *S. cerevisiae* ATCC 96581. The faster *in vivo* conversion rate of furfural and HMF in *S. cerevisiae* ATCC 96581 and the resulting shorter time for the cells being exposed to the inhibitors may be favorable for cell recovery.

The mechanisms of furan inhibition in yeasts are apparently very complex. In *S. cerevisiae*, transcriptome analysis revealed hundreds of genes that were differentially expressed in response to HMF (Ma and Liu 2010). In addition to the induced expression of genes responsible for direct HMF reduction such as the genes of ADH, genes involved in the pentose phosphate pathway (Gorsich et al. 2006), genes for degradation of damaged proteins and protein modifications, genes involved in response to osmotic stress, in DNA damage repair and nutrient starvation (Lin et al. 2009) are also found differentially expressed in response to HMF. Therefore, inhibition of *S. passalidarum* by furfural and HMF may also be due to some other unidentified damages caused by furans.

Finally, the presence of xylose showed slightly positive effects on both aerobic cell growth and anaerobic fermentation performance of *S. passalidarum* in medium with added furfural or HMF. In *Pichia stipitis* xylose reductase catalyzes reduction of both xylose and HMF (Almeida et al. 2008). This might be the similar case in *S. passalidarum*. Based on the results obtained with disrupted cells it can be concluded that cells grown in the presence of xylose possess a higher enzymatic capacity (two fold) to reduce furfural and HMF than do cells grown without xylose (Table 3.4). However, when cells were grown in the presence of furfural and HMF, no substantial positive influence of xylose on furfural/HMF reduction rate was detected (Table 3.3). Thus, it appears that big differences of enzyme levels in whole cells do not give such big difference on the reduction of furfural and HMF. One possible explanation could be that furans may have a lower affinity for *S.*

*passalidarum* xylose reductase than do xylose. This hypothesis can be tested by purification of the enzyme and investigate its enzymatic properties. However, this is outside the scope of this study.

In conclusion, the novel xylose-fermenting yeast *S. passadliarum* is sensitive to the inhibitors such as furfural and HMF formed after the pretreatment of lignocellulosic biomass, which prevents the immediate application of this yeast in the lignocellulosic bioethanol production. Therefore the inhibitor tolerance of this yeast needs to be improved. Further clarification of the metabolism and the inhibition mechanism of this yeast can favor the strain improvement.

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# Chapter 4

## Improved inhibitor tolerance in xylose fermenting yeast *Spathaspora passalidarum* by mutagenesis and protoplast fusion

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### 4.1 Abstract

The xylose fermenting yeast *Spathaspora passalidarum* showed excellent fermentation performance utilizing glucose and xylose under anaerobic conditions. But this yeast is highly sensitive to the inhibitors such as furfural present in the pretreated lignocellulosic biomass. In order to improve the inhibitor tolerance of this yeast, a combination of UV mutagenesis and protoplast fusion was used to construct strains with improved performance. Firstly, UV induced mutants were screened and selected for improved tolerance towards furfural. The most promising mutant, *S. passalidarum* M7, produced 50% more final ethanol than the wild type strain in a synthetic xylose medium containing 2 g/L furfural. However, this mutant was unable to grow in a medium containing 75% liquid fraction of pretreated wheat straw (WSLQ), in which furfural and many other inhibitors were present. Hybrid yeast strains, obtained from fusion of the protoplasts of *S. passalidarum* M7 and a robust yeast, *Saccharomyces cerevisiae* ATCC 96581, were able to grow in 75% WSLQ and produce ethanol at around 0.4 g ethanol/g consumed xylose. Among the selected hybrid strains, the hybrid FS22 showed the best fermentation capacity in 75% WSLQ. Phenotypic and molecular analysis indicated that *S. passalidarum* M7 was the dominant parental contributor to the hybrid. Rearrangement of DNA segments from the other parental strain *S. cerevisiae* ATCC 96581 possibly occurred in FS22. In summary, the hybrids are characterized by desired phenotypes

derived from both parents, namely the ability to ferment xylose from *S. passalidarum* and an increased tolerance to inhibitors from *S. cerevisiae* ATCC 96581.

## 4.2 Introduction

A recently discovered xylose fermenting yeast *S. passalidarum* (Nguyen et al. 2006) has shown a potential to be utilized in lignocellulosic ethanol production because of its excellent glucose and xylose fermentation capability under anaerobic conditions (Chapter 2). However, this yeast also exhibits low tolerance towards pretreatment-generated inhibitors such as furfural (Chapter 3).

The aim of this study is to improve the inhibitor tolerance of this microorganism. Since the mechanism of inhibitor tolerance and the genetic background of *S. passalidarum* remain unclear, a genome shuffling method combining mutagenesis and protoplast fusion was applied in the present study. This approach allows recombination of genomes and intergeneric transfer of genomic DNA that otherwise cannot happen naturally. It provides an alternative to the rational refined method for rapid strain improvement, which is especially important for the development of commercial strains. In this study, a hybrid yeast strain FS22 was developed through mutagenesis of *S. passalidarum* followed by protoplast fusion with another robust industrial yeast strain, *S. cerevisiae* ATCC 96581 (=TMB3000, Martín and Jönsson 2003). The fermentation performance of this hybrid yeast was substantially improved in a liquid fraction of hydrothermally pretreated wheat straw (Inbicon A/S, DONG Energy subsidiary, Denmark) containing numerous inhibitors.

## 4.3 Materials and Methods

### *Culture and selection media*

The culture and the selection media used in this study are listed below: (1) Yeast extract peptone dextrose (YPD) medium: 10 g/L yeast extract, 20 g/L peptone, and 30 g/L glucose; (2) Yeast extract

peptone xylose (YPX) medium: 10 g/L yeast extract, 20 g/L peptone, and 30 g/L xylose; (3) Yeast extract peptone xylose furfural (YPXF) medium: 10 g/L yeast extract, 20 g/L peptone, 30 g/L xylose and additional furfural as indicated; (4) Liquid fraction of pretreated wheat straw (WSLQ) medium: wheat straw was pretreated by a hydrothermal pretreatment method in the pilot plant of Inbicon A/S (DONG Energy subsidiary, Denmark) at 190°C for 12 minutes. After the pretreatment, the slurry was separated into solid and liquid part. The liquid fraction containing most of the inhibitors generated during the pretreatment was used as the inhibitory/selection medium with additional glucose and/or xylose as indicated. The composition of WSLQ was analyzed and described as following:  $0.54 \pm 0.01$  g/L furfural;  $0.29 \pm 0.01$  g/L HMF;  $2.86 \pm 0.28$  g/L total phenols;  $1.28 \pm 0.04$  g/L formic acid;  $3.39 \pm 0.08$  g/L acetic acid;  $0.61 \pm 0.01$  g/L lactic acid;  $1.72 \pm 0.03$  g/L free glucose and  $2.29 \pm 0.07$  g/L free xylose. 75% WSLQ medium was made by 75% WSLQ mixed with MilliQ water and additional glucose and/or xylose as indicated. YPD, YPX, YPXF and WSLQ agar plates were made by adding 20g/L agar into the medium.

### ***Yeast strains and culture conditions***

*S. cerevisiae* ATCC 96581 and *S. passalidarum* MYA-4345 were used in this study. The propagation and pre-culture preparation of the strains were according to the procedure described in Chapter 2.

### ***UV mutagenesis***

UV irradiation (254 nm) was performed in CL-1000 ultraviolet crosslinker-UVP (Buch & Holm A/S, Denmark). *S. passalidarum* was grown overnight in 5 ml YPX medium and collected by centrifugation at 4000g for 10 minutes. Survival curves were determined by spreading serial dilutions ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ) of cells on YPX agar plates after being exposed to different doses of UV



irradiation (1000, 2000, 3000, 4000, 5000  $\mu\text{J}/\text{cm}^2$ ). Colonies were counted after incubation at 30°C for 2 days. Percentage survival rates were calculated as follows: Percentage survival = [(CFU /ml of cells irradiated) / (CFU/ml of cells unirradiated)]  $\times$  100%, where CFU refers to Colony Forming Units.

UV mutagenesis for furfural-resistant *S. passalidarum* mutant was conducted by firstly collecting overnight-grown cells by centrifugation at 4000g for 10 minutes and washing the cells with 0.9% NaCl solution twice. The washed cells were re-suspended in 0.9% NaCl solution and adjusted to a concentration of  $1 \times 10^7$  cells/ml. 100  $\mu\text{l}$  of cells were spread on YPXF (YPX+2.5 g/L furfural) agar plates and exposed to 1500  $\mu\text{J}/\text{cm}^2$  UV radiation. The YPXF agar plates were then wrapped with aluminized foil immediately to avoid the photorepairs of UV-induced mutations in cells (Suter et al. 2000) and incubated at 30°C for 4 days. Colonies were transferred to YPX agar plates by replica plating. After incubation at 30°C for 24 hours, the colonies were transferred back to YPXF agar by replica plating. The replica plating between YPX agar and YPXF agar were repeated twice in order to select mutants with a stable resistance to furfural. The selected mutant strains were maintained on YPX agar plates for further characterization.

### ***Protoplast preparation and fusion***

The selected *S. passalidarum* mutant strain M7 and *S. cerevisiae* ATCC 96581 were used as parental yeast strains for protoplast fusion. Protoplasts were prepared according to a modified version of the method described by Wei et al. (2001). Cells grown overnight in YPD medium were harvested by centrifugation at 4000g for 10 minutes and washed twice by 0.2 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer (pH 5.8). The washed cells were re-suspended and pre-incubated in the same buffer containing 0.1% (w/v) EDTA and 0.1% (v/v) 2-mercaptoethanol at room temperature for 10 minutes. The pre-incubated cells were washed again in 0.2 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer (pH

5.8) and re-suspended in the same buffer to adjust the cell concentration to  $3 \times 10^6$  cells /ml. Lyticase (400 units) (Sigma) was added to the cell suspensions and cells were incubated for additional 30 minutes at 30°C to generate protoplasts. Samples were taken at an interval of 10 minutes and the formation of protoplast was evaluated by observing the morphology of the cells (spherical shapes) when exposed to hypotonic solution.

Equal numbers of protoplasts from both parental strains were collected and mixed in 1 ml of freshly prepared fusion solutions containing 40% PEG-8000 and 10 mM  $\text{CaCl}_2$  in Milli-Q water. The mixed protoplasts were incubated for 30 minutes at 30°C. The incubated protoplasts were collected by centrifugation in a table centrifuge and washed three times with 0.9% NaCl (w/v). The washed protoplasts were then incubated again in 5 ml regeneration medium (1 g/L  $\text{KH}_2\text{PO}_4$ , 0.5 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 90 g/L sorbital, 20 g/L glycerol, 10 g/L yeast extract, 20 g/L peptone and 30 g/L glucose) at 30°C for 6 hours. The incubated protoplasts were re-suspended in YPD liquid medium and spread on YPD agar plates. The plated cells were incubated at 30°C until visible colonies appear. The colonies were later transferred by replica plating to the agar plates containing 75% of the liquid fraction of pretreated wheat straw with addition of 30 g/L xylose (75% WSLQ+X). 75% WSLQ+X agar plates were incubated at 30°C for 4 days and the grown colonies were transferred back to YPX agar plates. The transfers between YPX and 75% WSLQ+X plates were repeated three times to select hybrid strains with stable tolerance to various inhibitors present in WSLQ. The finally selected colonies were maintained on YPX agar plates for further characterization.

### ***Anaerobic fermentation***

Mutant and hybrid yeast strains were transferred six times sequentially on YPX agar plates before being inoculated into liquid media for anaerobic fermentation. All anaerobic fermentations

were performed as batch fermentation using 50 ml working volume in 100 ml anaerobic vials. The residual oxygen in the medium and head space of the vial was not flushed in order to support initial cell growth. The vials were inoculated to an OD<sub>620nm</sub> of 0.1~0.2 and incubated at 32°C in an orbital shaker with the rotation speed of 120 rpm.

Samples for analysis were taken under sterile conditions. Cell growth during the fermentation was monitored by optical density at 620 nm using Ultrospec 3000 (Pharmacia Biotech, Sweden). Carbon recovery was calculated as described in Chapter 2.

### ***Chemical analysis***

Glucose, xylose, ethanol, glycerol, lactic acid, formic acid and acetic acid were analyzed by high performance liquid chromatography (HPLC) system (Shimadzu, Japan) using refractive index detector equipped with an Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) running at 63°C with 4 mM H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.6 ml/min. Glycerol was analyzed during the fermentation in synthetic medium (i.e. YPX and YPXF media) but not during fermentation in WSLQ medium containing formic acid which overlaps with glycerol in the chromatogram.

Furfural and HMF were analyzed by the same HPLC system using the UV lamp at 250 nm in a SPD-M10AVP diode-array detector equipped with an Aminex HPX-87H column running at 63°C with 4 mM H<sub>2</sub>SO<sub>4</sub> as eluent at a flow rate of 0.6 ml/min.

Total phenol contents in the liquid fraction of wheat straw were determined by Prussian Blue assay as described by Price and Butler (1977). Phosphoric acid was used for stopping the reaction and gum arabicum was used to prevent precipitation. The absorbances of samples were measured at 700 nm. Catechol solution was used as standard.

### ***Extraction and estimation of DNA content***

The nucleic acids of *S. passalidarum* mutant strain M7, *S. cerevisiae* ATCC 96581 and six selected yeast hybrids were extracted by using “Yeast Genomic DNA Isolation Kit” following the supplier’s instruction (Norgen Biotek Corporation, Canada). DNA content was determined by fluorometry using the protocol described in “DNA Quantitation Kit, fluorescence assay” (Sigma). Bisbenzimidazole, which binds primarily to AT sequences, was used as the fluorescent dye. The wavelengths of the exciting light and emission light for DNA quantitation were 360 nm and 460 nm, respectively. The number of the cells was determined by OD<sub>620nm</sub> calculated from linear correlations between OD<sub>620nm</sub> and cell number.

#### ***PCR amplification and restrictive digestion reactions***

PCR amplifications were conducted using the genomic DNA derived from *S. passalidarum* M7, *S. cerevisiae* ATCC 96581 and the hybrid strain FS22 as templates. Table 4.1 lists the primers used in this study. PCR reactions were carried out in a 50 µl reaction mixture containing 1×PCR buffer (Fermentas, Denmark), 2.5 mM MgCl<sub>2</sub>, 2 mM deoxynucleoside triphosphates (Fermentas, Denmark), 600 nM (each) primers (Eurofins MWG Operon, Germany), 0.1 µg of DNA template and 1 U of polymerase RUN (A&A Biotechnology, Poland). Amplification was performed by using Mycycler Thermal Cycler (Bio-Rad Laboratories Ltd., USA) under the standard conditions with 60°C as the annealing temperature.

The amplified PCR products were digested with the restriction enzymes FastDigest® *Hae*III and *Eco*RI (Fermentas, Denmark) in separate reactions in a total reaction volume of 30 µl containing 1× FastDigest® buffer, 0.2 µg PCR product, 1 µl FastDigest® enzyme and nuclease-free water. The DNA fragments generated were analyzed by 1% agarose gel electrophoresis. The gel was photographed with Biospectrum Imaging System (Ultra-Violet Products Ltd., UK).

**Table 4.1** Sequences of the primers and the amplified regions

Primer name	Sequence (5'→3')	Amplified region	Size of the amplified fragments (bp)
Spa5.8s_fr	GAACCTGCGGAAGGATCATTAC	5.8S ribosomal RNA, and the two internal transcribed spacer (ITS) regions in <i>S. passalidarum</i> ATCC MYA-4345	499
Spa5.8s_re	CTTAAGTTCAGCGGGTAGTCC		
Sac5.8s_fr	CAAGAGATGGAGAGTCCAGC	5.8S ribosomal RNA, and the two internal transcribed spacer (ITS) regions in <i>S. cerevisiae</i> ATCC 96581	669
Sac5.8s_re	CATTGTTTCGCCTAGACGCTC		

### ***In vitro* furfural and hydroxymethylfurfural reduction activity assay**

For the preparation of the cell-free extracts, yeast cells were grown overnight in YPX or YPDX medium as indicated. Cells were harvested from 10 ml of cultures by centrifugation at 4500 rpm for 10 minutes at 4°C. The cell pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.0), and the pellet was resuspended in 900 µl of the same buffer with the addition of 100 µl acid-washed glass beads (particle size: 425-600 µm, U.S. sieve). The cells were disrupted by bead beating using a Bullet Blender (Next Advance Inc., USA) for 10 minutes at 4°C. The final homogenates were centrifuged at 14000 rpm for 5 minutes at 2°C and the supernatants were used for enzymatic activity measurement. Protein concentrations in the cell-free extracts were determined by “Quick Start™ Bradford Protein Assay Kit” (Bio-Rad Laboratories Ltd., USA) following the manufactures instruction.

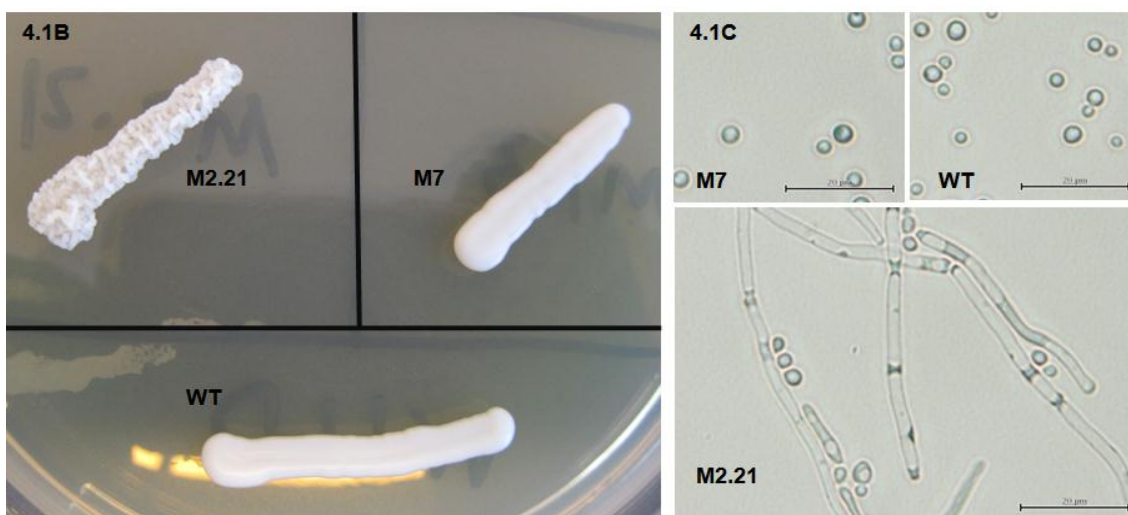
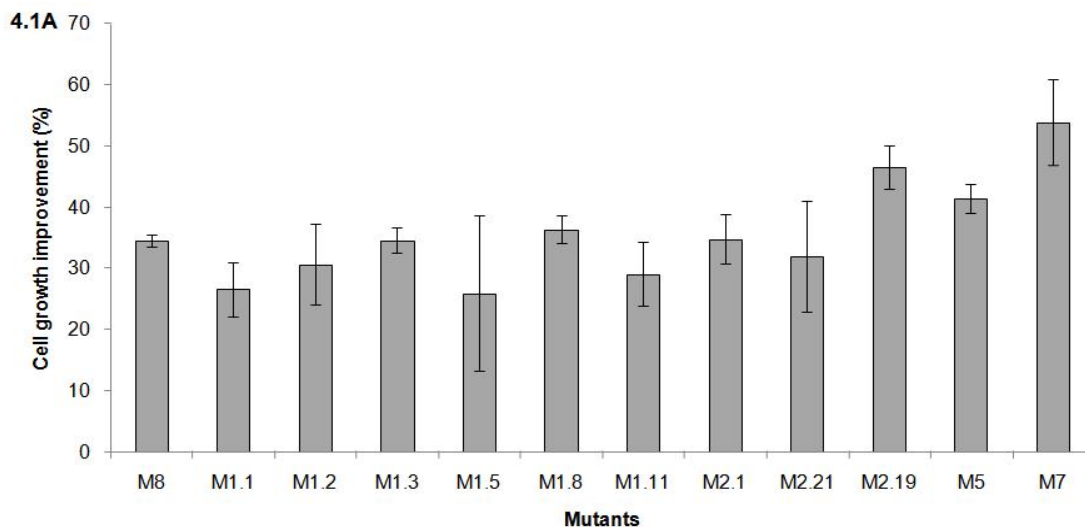
*In vitro* furfural and hydroxymethylfurfural (HMF) reduction activities were determined according to the procedure described in Chapter 3.

## **4.4 Results**

### ***UV induced S. passalidarum mutants***

Winston (2008) has recommended choosing the UV dosage leading to 40% ~ 70% survivals for an efficient mutagenesis. Therefore UV radiation at a dosage of 1500 µJ/cm<sup>2</sup>, which leads to 45%

of the survival cells as determined from survival curve (Appendix Figure A2), was used in the UV mutagenesis to generate furfural-resistant mutants. After UV radiation at this dosage, 184 colonies were recovered from YPX<sub>F</sub> agar plates containing 2.5 g/L furfural after incubation at 30°C for 4 days. These colonies were transferred to YPX plates to relieve the selection pressure and then transferred back to the YPX<sub>F</sub> plates. Finally, 17 colonies were recovered from YPX<sub>F</sub> plate after three cross transfers between YPX and YPX<sub>F</sub> agar plates. These colonies were tested again in YPX<sub>F</sub> liquid medium containing 2 g/L furfural and 12 of the colonies showed enhanced cell growth relative to the wild type, calculated from 25% to 55% of cell growth improvement after 96 hours of anaerobic fermentation (Figure 4.1A). The colony phenotype of most mutants were similar to that of the wild type *S. passalidarum*, except for the mutant strains M1.5 and M2.21, which displayed rough colony surfaces (Figure 4.1B, M2.21 as example). The cellular morphology of wild type *S. passalidarum* and most mutants were found round and globular (Figure 4.1C, WT and M7). However, elongated cells with septate hyphae were dominant in mutants M1.5 and M2.21, leading to the rough and curly colony surfaces (Figure 4.1C, M2.21 as example). Unlike *S. passalidarum* wild type which showed septate hyphae after 7 days on the corn meal (CM) agar (Nguyen et al. 2006), the mutant strains M1.5 and M2.21 showed septate hyphae in less than 24 hours of growth on YPX agar plates (data not shown).



**Figure 4.1** Cell growth improvement and the representative morphology of UV induced mutants of *S. passalidarum*. **4.1A**: Growth enhancements of mutants of *S. passalidarum* after 96 hours of anaerobic fermentation in YPXF medium containing 2 g/L furfural. Cell growth improvement (%) =  $[\text{OD}_{620\text{nm}}(\text{mutant}) - \text{OD}_{620\text{nm}}(\text{wild type})] / \text{OD}_{620\text{nm}}(\text{wild type}) \times 100\%$ .  $\text{OD}_{620\text{nm}}(\text{wild type}) = 0.74 \pm 0.14$ . Data presented are the average of triplicate experiments and error bars indicate the standard deviations. **4.1B-C**: Representative plate morphology and cellular morphology of wild type *S. passalidarum* (WT), mutant strains M7 and M2.21 on YPX agar plates after 48 hours of incubation at 30°C. Scale bar in **4.1C** is 20μm.

### *Anaerobic fermentation of UV induced mutants*

Anaerobic fermentation performed with *S. passalidarum* wild type and UV induced mutants in YPXF liquid medium containing 2 g/L furfural showed the most enhanced growth in mutants M7, M2.19 and M5 (Figure 4.1A). Figure 4.2 shows the representative growth curves of wild type *S. passalidarum* and the two selected mutants M5 and M7 during anaerobic fermentation in YPX

medium (Figure 4.2A) and in YPXF medium containing 2 g/L furfural (Figure 4.2B). In the latter medium, both of the mutants showed the overall enhanced growth and higher final cell density as compared with the wild type strain. Moreover, xylose consumption, ethanol yield, and final ethanol concentration were also elevated with the mutant strains during the fermentation of furfural containing substrate, as compared with the wild type (Table 4.2). The average furfural reduction rates in the two mutant strains were higher than that in the wild type *S. passalidarum* within 72 hours (Table 4.2). The increased *in vitro* NADH preferred furfural and hydroxymethylfurfural (HMF) reduction activities were also observed in the two mutant strains as compared with the wild type (Figure 4.3). *S. passalidarum* M7 was finally selected as the mutant strain exhibiting the highest tolerance to furfural based on its performance on the selection medium. Further screening on 75% WSLQ medium, which contains furfural and numerous other inhibitors, showed complete growth inhibition on all 12 selected furfural-resistant mutant strains.

**Table 4.2** Anaerobic fermentation of *S. passalidarum* wild type and UV induced mutants M5 and M7 in YPXF medium containing 2 g/L furfural

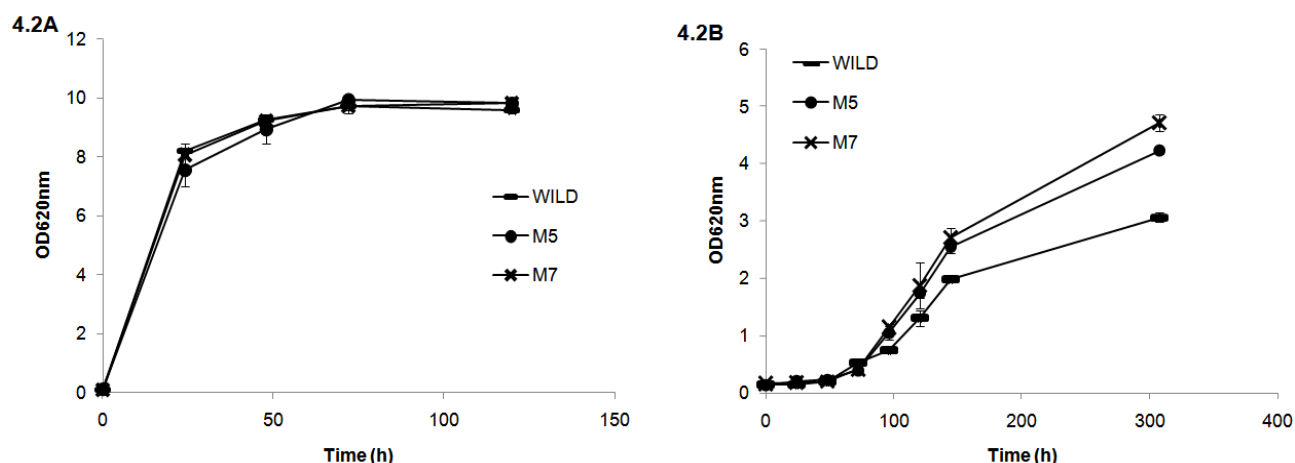
Strain	Xylose consumption (%) <sup>a</sup>	Final ethanol concentration (g/L) <sup>a</sup>	Ethanol yield (g/g consumed xylose) <sup>a</sup>	Average furfural reduction rate (g/L/h) <sup>b</sup>	Carbon recovery (%) <sup>a</sup>
Wild type	24.5 ± 0.9	2.85 ± 0.08	0.38 ± 0.01	0.009 ± 0.001	89.7 ± 1.4
M5	29.5 ± 0.4	3.31 ± 0.02	0.38 ± 0.02	0.012 ± 0.001	91.3 ± 2.2
M7	36.9 ± 1.5	4.23 ± 0.43	0.40 ± 0.04	0.016 ± 0.001	91.6 ± 4.9

<sup>a</sup>. Values presented were calculated after 308 hours of anaerobic fermentation.

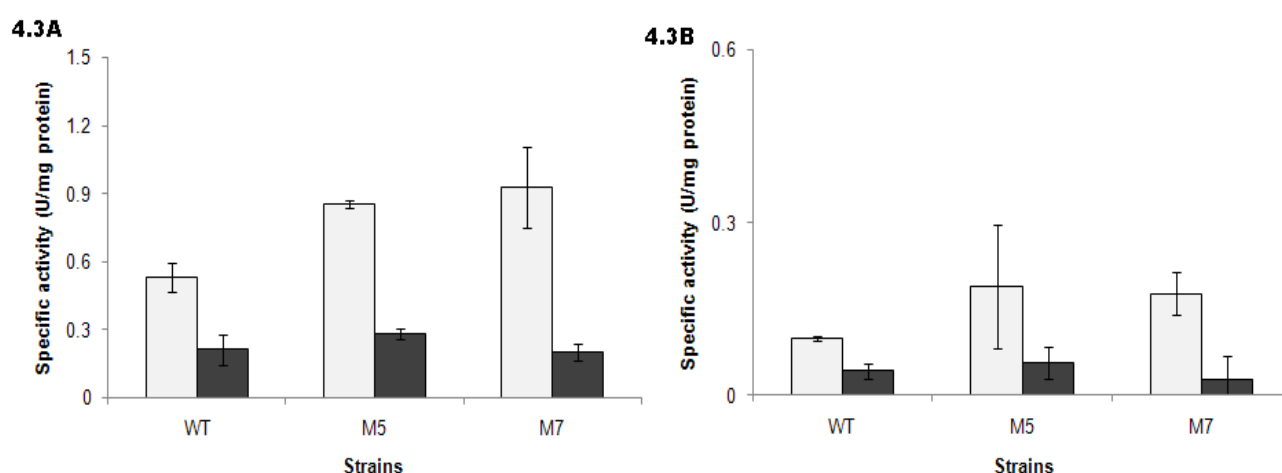
<sup>b</sup>. Average furfural reduction rates were calculated after 72 hours with few amounts of furfural still left in the media.

<sup>a+b</sup> Data presented are average of duplicate experiments ± the ranges of measurements.





**Figure 4.2** Time course of the growth of wild type *S. passalidarum* and two UV-induced mutant strains (M5 and M7) under anaerobic conditions in YPX medium (4.2A) and YPXF medium containing 2 g/L furfural (4.2B); wild type (-), M5 (filled circle), M7 (x). Data presented are average of duplicate experiments and error bars indicate the ranges of measurements .



**Figure 4.3** *In vitro* furfural (4.3A) and hydroxymethylfurfural (HMF) (4.3B) reduction activities measured from the cell-free extracts of *S. passalidarum* wild type strain (WT) and mutants (M5 and M7) using NADH or NADPH as co-factor; NADH (light column), NADPH (dark column). Yeast strains were anaerobically incubated in YPX liquid medium for 14 hours before the measurement. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1  $\mu$ mol of NAD(P)H per minute using either furfural or HMF as substrate. Data presented are average of three independent measurements and error bars indicate the standard deviations.

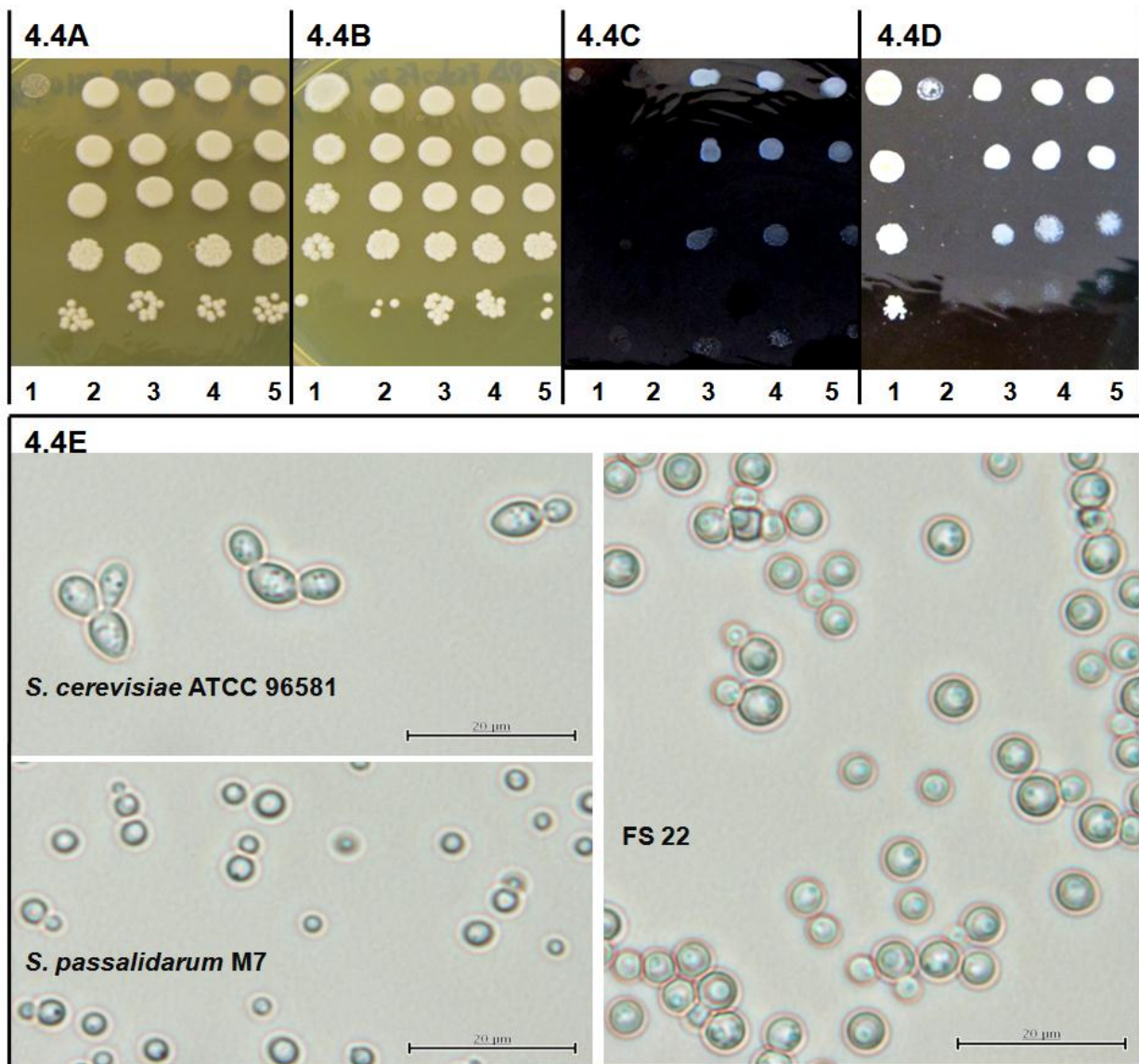
### Hybrids of *S. passalidarum* and *S. cerevisiae* by protoplast fusion

In order to further improve the inhibitor tolerance of the UV induced *S. passalidarum* mutant strain M7, protoplast fusion were carried out between this strain and another robust industrial yeast strain *S. cerevisiae* ATCC 96581 (=TMB3000, Martín and Jönsson 2003). *S. cerevisiae* ATCC

96581 showed the complete glucose consumption and efficient ethanol production during anaerobic fermentation of 75% WSLQ with addition of 30g/L glucose (data not shown). However, *S. cerevisiae* ATCC 96581 failed to grow when xylose was added to 75% WSLQ as the only carbon source. Thus, 75% WSLQ with addition of 30 g/L xylose (75%WSLQ+X) was used as selection substrate for potential hybrids. During the selection process, the colonies grown on 75%WSLQ+X agar plates were transferred to YPX agar plates to release the selection pressure from inhibitors present in WSLQ. The colonies grown on YPX were transferred back again to the 75%WSLQ+X agar plates. This cross transfer was repeated three times. The number of the colonies able to grow on 75%WSLQ+X agar plates decreased from 141 to 24 after the third transfer. These 24 colonies were selected as putative yeast hybrids and named as from FS1 to FS24.

Figure 4.4 shows the morphology of the parental strain *S. passalidarum* M7 and *S. cerevisiae* ATCC 96581, as compared with three putative hybrid strains (FS2, FS8 and FS22) on the agar plates of YPX (Figure 4.4A), YPD (Figure 4B), 75% WSLQ with additional 30 g/L xylose (75%WSLQ+X) (Figure 4.4C) and 75% WSLQ with additional 30 g/L glucose (75%WSLQ+D) (Figure 4.4D). The results showed that *S. cerevisiae* ATCC 96581 was unable to grow on YPX and 75%WSLQ+X agar plate (Figure 4.4A and 4.4C, Lane 1), due to its inability to utilize xylose. However, *S. cerevisiae* ATCC 96581 showed efficient growth on 75%WSLQ+D agar plate (Figure 4D, lane 1), indicating its high tolerance to the inhibitors present in WSLQ. As a comparison, *S. passalidarum* M7 was able to grow on both YPX and YPD agar plates (Figure 4.4A and 4.4B, lane 2), but was unable to grow on 75% WSLQ (Figure 4.4C and 4.4D, lane 2), indicating that the strain was able to utilize both glucose and xylose but was sensitive to the inhibitors present in WSLQ. In contrast to the two parental strains, all the presented three putative hybrid yeast strains were able to grow on all the tested agar plates (Figure 4.4A-D, lane 3-5), although with noticeably weaker growths on WSLQ agar plates (Figure 4.4C and 4.4D, lane 3-5), as characterized by thinner cell

layer, smaller colony size and longer incubation time, compared with those grown on the rich YPD and YPX agar plates (Figure 4.4A and 4.4B, lane 3-5). The cell size of the hybrid yeast FS22 was bigger than parental *S. passalidarum* M7 but smaller than parental *S. cerevisiae* ATCC 96581 (Figure 4.4E).



**Figure 4.4 A-D:** Ten-fold serial dilutions spotting of *S. cerevisiae* ATCC 96581 (**Lane 1**), *S. passalidarum* M7 (**Lane 2**) and putative hybrid yeast strains FS2 (**Lane 3**), FS8 (**Lane 4**), and FS22 (**Lane 5**) on YPX (**4.4A**), YPD (**4.4B**), 75% WSLQ+X (**4.4C**) and 75% WSLQ+D (**4.4D**) plates. Pictures were taken after incubation at 30°C for 2 days (**4.4A** and **4.4B**) and 4 days (**4.4C** and **4.4D**); **4.4E:** Cellular morphologies of *S. passalidarum* M7, *S. cerevisiae* ATCC 96581 and the hybrid strain FS22 after 7 days of incubation on YPD agar plates at 30°C. Scale bar is 20µm.

### ***Molecular analysis of protoplasts fused hybrids***

The DNA contents of six putative hybrid strains and the parental strains of *S. passalidarum* M7 and *S. cerevisiae* ATCC 96581 were quantified (Table 4.3). The results showed that all putative hybrids contained different quantities of DNA, but all values were higher than that in the parental strain *S. passalidarum* M7 and lower than or around that in the parental strain *S. cerevisiae* ATCC 96581 (Table 4.3).

To further analyze the putative yeast hybrids, PCR amplification was performed with the chromosomal DNA from parental and hybrid strains as templates and afterwards subjected to restrictive enzymatic digestion. Primers specific for *S. passalidarum* 5.8S ribosomal rRNA and the two internal transcribed spacer (ITS) regions were used in the PCR reactions and amplified around 700bp, 500bp and 500bp fragments from chromosomal DNA of *S. cerevisiae* ATCC 96581 (Figure 4.5A, Lane 1), *S. passalidarum* M7 (Figure 4.5A, Lane 2) and the putative hybrid FS22 (Figure 4.5A, Lane 3), respectively. Restrictive enzyme digestion of the above PCR amplified fragments showed the different patterns in *S. cerevisiae* ATCC 96581 (Figure 4.5B, Lane 1H) from those in *S. passalidarum* M7 and FS22 (Figure 4.5B, Lane 2H and Lane 3H). The restriction patterns of the PCR fragments from *S. passalidarum* (Figure 4.5B, Lane 2H) and hybrid FS22 (Figure 4.5B, Lane 3H) were however the same (a 99 bp fragment and a 400 bp fragment after digestion by *Hae*III). The lengths of the amplified fragments (Figure 4.5A, Lane 2 and Lane 3) and the digestion patterns (Figure 4.5B, Lane 2H and 3H) were as expected, based on the DNA sequence of the amplified region of 5.8S rRNA-ITS in *S. passalidarum*. This indicated the presence of 5.8S rDNA of *S. passalidarum* in the hybrid FS22. The PCR fragment amplified from *S. cerevisiae* ATCC 96581 using the same set of primers is unknown due to the different size and restrictive patterns.

Moreover, primers specific for *S. cerevisiae* 5.8S rRNA-ITS region sequence were used in the PCR reactions and amplified a fragment around 600~700 bp from the chromosomal DNA of both *S.*

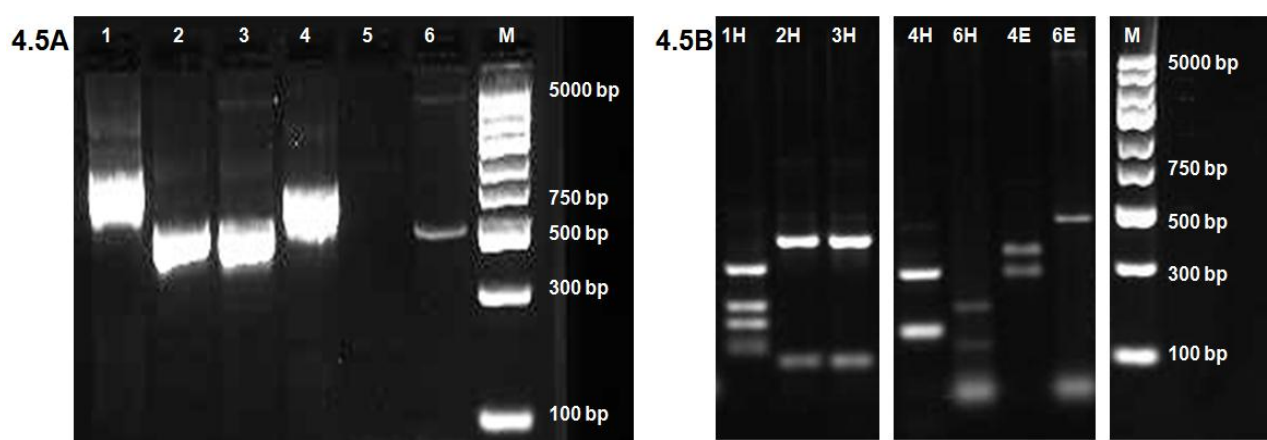
*cerevisiae* ATCC 96581 (Figure 4.5A, Lane 4) and the hybrid strain FS 22 (Figure 4.5A, Lane 6), while the control using *S. passalidarum* M7 chromosomal DNA as template failed to produce any PCR products (Figure 4.5A, Lane 5). However, although the length of the amplified fragments from *S. cerevisiae* ATCC 96581 and FS 22 chromosomal DNA were similar, the restrictive patterns of the two fragments (Figure 4.5B, Lane 4H compared with Lane 6H, and Lane 4E compared Lane 6E) were different. The restrictive pattern of the fragments from *S. cerevisiae* ATCC 96581 was as expected according to its DNA sequence (Figure 4.5B, Lane 4H: two overlapping fragments of 162 bp and 172 bp, and a 311 bp fragment after *Hae*III digestion; Lane 4E, a 304 bp fragment and a 365 bp fragment after *Eco*RI digestion). These results demonstrated that the hybrid FS22 contains a DNA segment that is not in *S. passalidarum* but is also different from 5.8S rRNA-ITS regions in *S. cerevisiae*, indicating the possible gene rearrangement happened in FS22. Similar situations were observed when PCR amplification specific for *S. cerevisiae* ALD5 and SFA1 gene regions were conducted. Different sizes of fragments were amplified from FS22 and *S. cerevisiae*, while no fragment was produced using *S. passalidarum* M7 chromosomal DNA as template (Appendix Figure A3, information of the corresponding PCR primers and amplified regions are listed in Appendix Table A1).

PCR amplification specific for ALD4, ADH6, and ADH7 gene regions in *S. cerevisiae* failed to produce any fragment from FS22 or *S. passalidarum* M7 chromosomal DNA (information of the corresponding PCR primers and amplified regions are listed in Appendix Table A1).

**Table 4.3** DNA contents of parental strains and six putative hybrid yeast strains

Strain	DNA content ( $10^{-6}$ ng/cell) <sup>a</sup>
<i>S. passalidarum</i> M7	$5.6 \pm 0.3$
<i>S. cerevisiae</i> ATCC 96581	$9.0 \pm 0.2$
FS1	$8.8 \pm 0.6$
FS2	$7.1 \pm 0.6$
FS8	$9.3 \pm 0.8$
FS14	$8.9 \pm 0.4$
FS19	$7.0 \pm 0.4$
FS22	$8.4 \pm 0.7$

<sup>a</sup> Data presented are the average of two independent measurements  $\pm$  the range of measurements

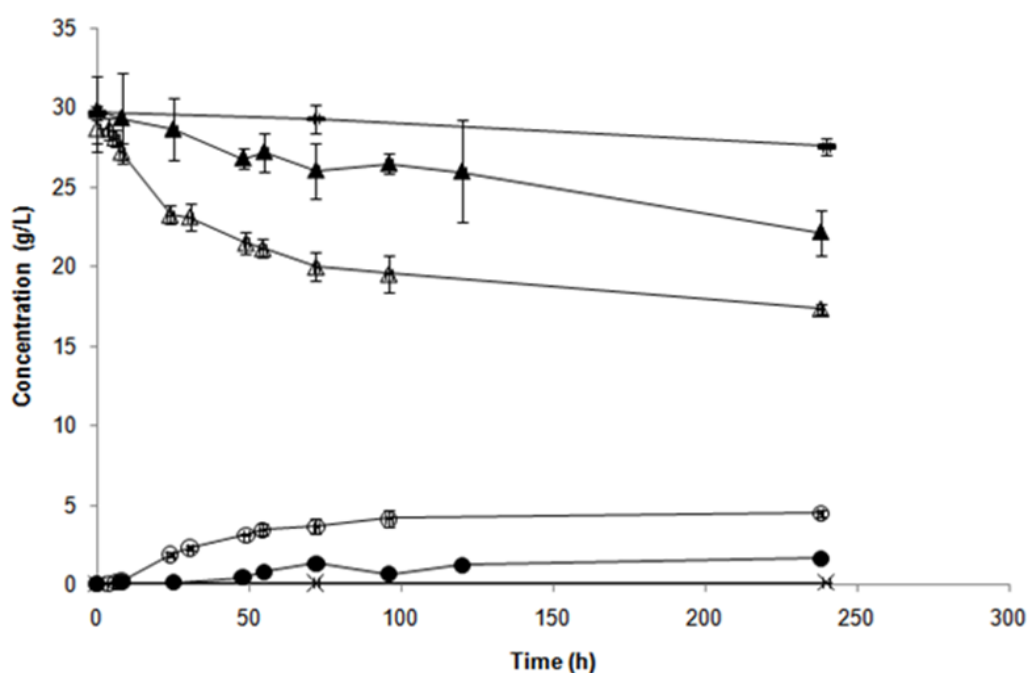


**Figure 4.5** Molecular analysis of putative yeast hybrid FS22 derived from protoplasts fusion of *S. cerevisiae* ATCC 96581 and *S. passalidarum* M7. **4.5A:** 5.8s rRNA PCR amplification reactions by using chromosomal DNA as templates derived from *S. cerevisiae* ATCC 96581, *S. passalidarum* M7 and hybrid strain FS22. **Lane 1:** *S. cerevisiae* chromosomal DNA as template using primers of Spa5.8s\_fr and Spa5.8s\_re; **Lane 2:** *S. passalidarum* chromosomal DNA as template using primers of Spa5.8s\_fr and Spa5.8s\_re; **Lane 3:** FS22 chromosomal DNA as template using primers of Spa5.8s\_fr and Spa5.8s\_re; **Lane 4:** *S. cerevisiae* chromosomal DNA as template using primers of Sac5.8s\_fr and Sac5.8s\_re; **Lane 5:** *S. passalidarum* chromosomal DNA as template using primers of Sac5.8s\_fr and Sac5.8s\_re; **Lane 6:** FS22 chromosomal DNA as template using primers of Sac5.8s\_fr and Sac5.8s\_re; **Lane M:** GeneRuler™ Express DNA ladder (Fermentas, Denmark); **4.5B:** Restriction analysis of the fragments shown in 4.5A using restriction enzymes *Hae*III and *Eco*RI. Lane sequences were rearranged after electrophoresis of the fragments in the same gel. **Lane 1H:** Product from Lane 1 in 5A digested by *Hae*III; **Lane 2H:** Product from Lane 2 in 5A digested by *Hae*III; **Lane 3H:** Product from Lane 3 in 5A digested by *Hae*III; **Lane 4H:** Product from Lane 4 in 5A digested by *Hae*III; **Lane 6H:** Product from Lane 6 in 5A digested by *Hae*III; **Lane 4E:** Product from Lane 4 in 5A digested by *Eco*RI; **Lane 6E:** Product from lane 6 in 5A digested by *Eco*RI; **Lane M:** GeneRuler™ Express DNA ladder (Fermentas, Denmark)

### Anaerobic fermentation of protoplasts fused hybrids

Anaerobic fermentation of the hybrid strains FS2 and FS22 showed the improved performance in 75% WSLQ medium with addition of 30 g/L xylose (Table 4.4 and Figure 4.6), as compared with

the parental strain *S. passalidarum* M7 which was unable to survive under the same condition. The hybrid strains FS2 and FS22 were able to grow and produce ethanol at a yield of 0.4 g/g consumed xylose. The strain FS22 showed a better fermentation performance than strain FS2, producing 90% more ethanol after 240 fermentation hours. However, the selected hybrid FS22 was still unable to consume all the sugars during the fermentation, with 60% of xylose left unfermented (Table 4.4 and Figure 4.6). Compared with the parental strain *S. passalidarum* M7, *in vitro* furfural and hydroxymethylfurfural (HMF) reduction activities were not significantly enhanced in the hybrid FS22 (Figure 4.7).



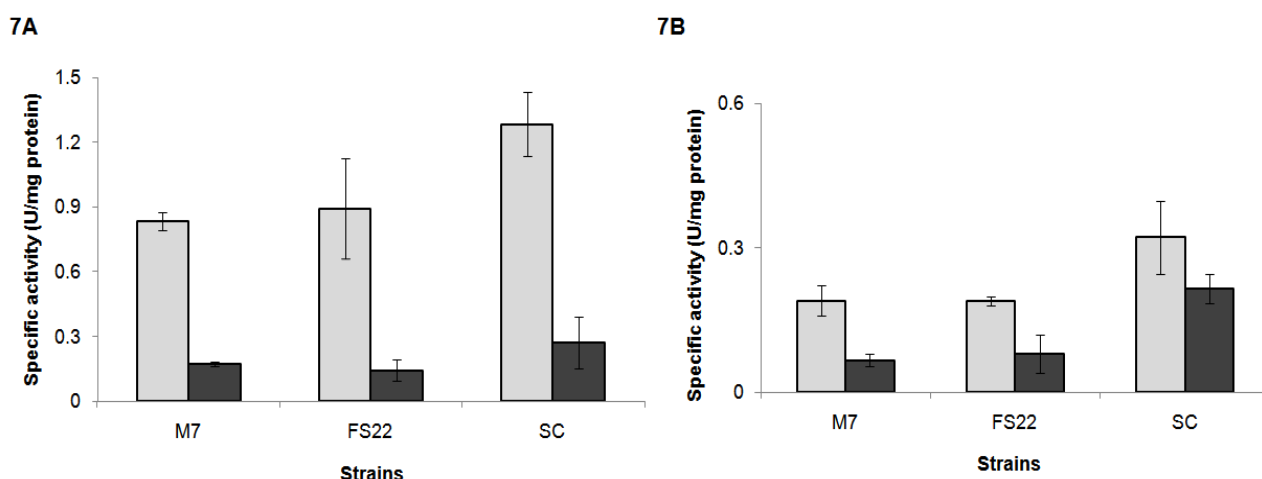
**Figure 4.6** Anaerobic fermentation of parental strain *S. passalidarum* M7, hybrid strains FS2 and FS22 in 75% WSLQ medium with addition of 30 g/L xylose; xylose consumed by *S. passalidarum* M7 (-), xylose consumed by FS2 (filled triangle), xylose consumed by FS22 (open triangle), ethanol produced by *S. passalidarum* M7 (x), ethanol produced by FS2 (filled circle), ethanol produced by FS22 (open circle). Data presented are average of triplicate experiments and error bars indicate the standard deviations.

**Table 4.4** Anaerobic fermentation of two hybrid yeast strains in 75% WSLQ medium with addition of 30 g/L xylose

Strain <sup>a</sup>	Xylose consumption (%) <sup>b</sup>	Ethanol yield (g/g xylose) <sup>b</sup>	Final ethanol concentration (g/L) <sup>b</sup>	Carbon recovery (%) <sup>b</sup>
FS2	21.5 ± 5.3	0.38 ± 0.05	2.4 ± 0.5	90.0 ± 7.8
FS22	39.4 ± 2.2	0.40 ± 0.03	4.5 ± 0.2	90.8 ± 6.5

<sup>a</sup>. Both parental yeast strains, *S. passalidarum* mutant M7 and *S. cerevisiae* ATCC 96581, cannot survive under the same condition.

<sup>b</sup>. Values are calculated from triplicate experiments after 240 hours of anaerobic fermentation ± standard deviations.



**Figure 4.7** *In vitro* furfural (4.7A) and hydroxymethylfurfural (HMF) (4.7B) reduction activities measured from the cell-free extracts of *S. passalidarum* M7 (M7), *S. cerevisiae* ATCC 96581 (SC) and hybrid yeast FS22 (FS22) using NADH or NADPH as co-factor; NADH (light column), NADPH (dark column). Yeast strains were anaerobically incubated in YPD liquid medium for 14 hours before the measurement. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of NAD(P)H per minute using either furfural or HMF as substrate. Data presented are average of three independent measurements and error bars indicate the standard deviations.

## 4.5 Discussion

The recently discovered xylose-fermenting yeast *S. passalidarum* (Nguyen et al. 2006) showed excellent performance on anaerobic fermentation of glucose and xylose (Chapter 2), and this is an important trait of this yeast, since most xylose-fermenting yeasts such as *Pichia sp.* and *Candida sp.* require costly and technically difficult microaerophilic conditions for xylose fermentation (Skoog



and Hahn-Hägerdal 1990; Keller et al. 1998). However, *S. passalidarum* is sensitive to inhibitors present in pre-treated lignocellulose hydrolysate such as furans, weak acids and phenolic compounds. This undesired low tolerance prevents *S. passalidarum* from being applied in industrial lignocellulosic bioethanol production. In the present study, it is shown that the inhibitor tolerance of *S. passalidarum* could be improved by a combination of mutagenesis and protoplast fusion, demonstrating that this strategy constitutes a route to improve the desirable traits of microorganisms even when the genetic constitution is unknown.

The inhibitors present in pretreated lignocellulosic hydrolysate can be grouped into furans, weak acids and phenolic compounds (Palmqvist and Hahn-Hägerdal 2000). Furfural is identified as a key toxin and correlates with the toxicity of the pretreated hydrolysate (Heer and Sauer 2008). Several strains of *S. cerevisiae* have been adapted to synthetic media with increasing concentrations of furfural and showed the improved fermentation performance in a lignocellulosic biomass based substrate (Heer and Sauer 2008; Keating et al. 2006). In the present study, the UV induced mutant strains of *S. passalidarum* such as M5 and M7 showed significantly improved fermentation capability in the synthetic medium (YPXF) with addition of 2 g/L furfural (YPXF). However, none of these mutant strains were able to survive in 75% WSLQ medium, in which the furfural concentration was only 0.4 g/L. This indicates that other compounds present in 75% WSLQ medium, e.g. formic acid (1.0 g/L), acetic acid (2.5 g/L), lactic acid (0.5 g/L), and/or total phenols (2.7 g/L) may cause considerable inhibition of the fermentation capacity of *S. passalidarum*. The adverse effects of furfural and HMF together with a variety of phenolic and aromatic compounds were reported (Mussatto and Roberto 2004; Delgenes et al. 1996). Similarly, the combination of furfural and acetic acid has caused greater reduction on cell growth of *S. cerevisiae* than the sum of the reductions caused by the individual compounds (Palmqvist and Hahn-Hägerdal 2000). Thus, although the furfural concentration in 75% WSLQ medium (0.4 g/L) was much lower than the

concentration that the mutant strains can tolerate in YPXF medium (2 g/L furfural), the action of furfural together with the other inhibitors present in the WSLQ substrate may additively or perhaps more likely, synergistically inhibit the growth of the mutant *S. passalidarum* strains under anaerobic conditions.

In order to further develop strains with improved performance in actual lignocellulosic bioethanol production, protoplast fusion between the most furfural-resistant mutant of *S. passalidarum* M7 and a robust yeast strain *S. cerevisiae* ATCC 96581 was carried out. The selected hybrid yeast strains were found able to grow and produce ethanol in 75% WSLQ medium with addition of 30 g/L xylose, and among these hybrid strains, FS22 showed the best performance. DNA content analysis among the different hybrid strains revealed variable quantities of DNA, but all exceeding that of *S. passalidarum* M7. This suggests that, at various efficiencies, DNA incorporation took place in the hybrid strains. It has been previously noted that heterokaryotic strains can be induced to fuse, but the products may be unstable and segregate to give various recombinant progeny (Fournier et al. 1977). The unstable heterokaryotic fusion products can only be maintained by application of a selection pressure, in the absence of which segregation to the parental genomes or various recombinant progeny will take place (Fournier et al. 1977). Fusion products can be stabilized through random loss of chromosomes or chromosome rearrangement (Klinner and Bottcher 1985). In the present study, the colonies grown on WSLQ agar plates were all transferred back to YPX plate three times to release the selection pressure, and the finally selected hybrid strains were sequentially transferred six times on YPX agar for proliferation. Different degrees of chromosome elimination might happen when the selection pressure disappeared or during the self-stabilization process of the hybrid strains. Moreover, cytoplasmic DNA incorporations in addition to nuclear rearrangements can not be excluded since the transfer of

cytoplasmic DNA, e.g. mitochondrial DNA occurs at a greater frequency than nuclear fusion in yeast (Curran and Carter 1989).

Molecular analysis of the hybrid strains revealed that *S. passalidarum* M7 was the dominant contributor to the genetic complement of the hybrids, while the chromosomal fragments from *S. cerevisiae* ATCC 96581 were possibly present in the hybrid strains. DNA rearrangement of these fragments possibly occurred during the stabilization process in the hybrids. Hybrids consisting of the nuclear genome of one parent plus a portion of that from the other have previously been demonstrated. For example, hybrids produced by the fusion of protoplasts of *Schizosaccharomyces pombe* and *Schizosaccharomyces octosporus* were demonstrated to have *S. octosporus* as the main genome contributor (Sipiczki et al. 1985). Likewise, hybrids resulting from the fusion of protoplasts of *Candida boidinii* and *Candida tropicalis* were found to consist of the genome from the former parent but with a few chromosomes from the latter parent (Kobori et al. 1991). The preferential retention of DNA from *S. passalidarum* in the selected hybrids may also be due to the selection process applied. The originally selected colonies from WSLQ agar plates were transferred back to YPX agar plates to release the selection pressure from the inhibitors while still keeping the selection pressure for xylose utilization. If the selection pressure was designed in a different way, the selected hybrids could have had different DNA retention and properties.

The mechanisms resulting in the sensitivity of *S. passalidarum* to the inhibitors are not clear. The increased furfural reduction activity in the UV induced mutant strains, M5 and M7, could contribute to their increased tolerance to the furfural as compared with the wild type. However, no further enhancement of furfural reduction activity has been detected in the most robust hybrid strain FS22 relative to the parental strains. Several enzymes such as alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALD) have been demonstrated to be correlated with the furan tolerance of yeasts (Larroy et al. 2002a, Larroy et al. 2002b, Aranda and del Olmo 2003). However, the

increased inhibitor tolerance in the hybrid yeast strains is not found related to ADH6, ADH7 or ALD4, ALD5 present in *S. cerevisiae* as suggested from PCR reaction analysis. Other mechanisms related to inhibitor tolerance include the changes in membrane integrity, membrane fluidity, organization and the dynamic structure of membrane lipids, which would all affect the cell's tolerance to toxic compounds. For example, the activity of the membrane H<sup>+</sup>-ATPase is important on counteracting weak acid stress (Almeida et al. 2007), and the membrane composition and its structure also correlates with the resistance of whole cells to toxic organic compounds (Heipieper et al. 1994). Since the hybrid strain FS22 has a different cell morphology as compared with *S. passalidarum* (bigger cell size and higher intention of aggregation), the membrane composition and structure in this yeast hybrid might be changed, which could contribute to the increased inhibitor tolerance of this strain.

In summary, the combination of UV induced mutations and protoplast fusion has produced *S. passalidarum* and *S. cerevisiae* hybrids with greatly improved anaerobic fermentation capacities on utilizing pretreated wheat straw. The hybrids are characterized by desired phenotypes derived from both parents, namely the ability to ferment xylose from *S. passalidarum* and an increased tolerance to inhibitors from *S. cerevisiae*. To our knowledge, this is the first demonstration of hybrid yeast between *S. passalidarum* and *S. cerevisiae*. However, even though the inhibitor tolerance has been improved significantly through mutagenesis and protoplast fusion, the most robust hybrid F22 was still not able to ferment all the xylose in the 75% WSLQ medium. The use of this strain alone to ferment lignocellulosic hydrolysate is therefore not practical. However, the possibility of using the combination of mutagenesis and intergeneric yeast protoplast fusion to create the novel strains embodying a range of characteristics from the parents have been demonstrated and may serve as an inspiration to further improve the xylose fermenting yeasts.

## 4.6 References

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# Chapter 5

## Ethanol production from un-detoxified pretreated lignocellulosic biomass by C6 and C5 fermenting yeasts

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### 5.1 Abstract

The fermentation performances of *Saccharomyces cerevisiae* ATCC 96581, *S. cerevisiae* Ethanol Red and *Spathaspora passalidarum* in media containing different concentrations of liquid fraction of pretreated corn stover (CSLQ) were compared, in order to study their tolerance to inhibitors present in the pretreated lignocellulosic biomass. *S. cerevisiae* ATCC 96581 was the most robust strain while the xylose fermenting yeast *S. passalidarum* was the most sensitive one. Adaptation of *S. cerevisiae* ATCC 96581 by sequentially transferring the strain to media with increasing contents of CSLQ further improved the inhibitor tolerance of this strain. The adapted strain completely fermented glucose in 100% CSLQ and the ethanol yield was 0.48 g/g glucose, while the parental strain was completely inhibited under this condition. The toxicity of the liquid fraction of corn stover (CSLQ) and wheat straw (WSLQ) were also compared. WSLQ appeared to be less toxic than CSLQ, although these two kinds of biomass were pretreated under the same conditions. Hybrid xylose fermenting strains FS2 and FS22 with improved inhibitor tolerance were obtained through mutagenesis of *S. passalidarum* and protoplast fusion with *S. cerevisiae* ATCC 96581 (Chapter 4). Co-fermentation of FS2 or FS22 with the adapted *S. cerevisiae* ATCC 96581 improved the final ethanol yield from total sugars by 11% and 26%, respectively, due to partial conversion of xylose into ethanol by the two hybrids. This work shows the prospect of a co-fermentation strategy using one robust C6 fermenting yeast for detoxification and simultaneous

glucose fermentation and a C5 fermenting yeast for converting xylose into ethanol. The present study is the first that describes the fermentation performance of *S. passalidarum* in the real lignocellulosic hydrolysate.

## 5.2 Introduction

The costs of lignocellulosic bioethanol production need to be reduced in order to make this sustainable clean energy source affordable on the global market. The toxicity of the pretreated lignocellulosic hydrolysate towards microorganisms and the presence of un-fermented pentose are two of the major challenges to be addressed in order to achieve the cost reduction.

Due to the harsh conditions (e.g. high pressure, high temperature, and addition of acids or oxidants) that are commonly applied in the present pretreatment methods, a broad range of inhibitors are generated. These pretreatment-generated inhibitors are mainly furans, weak acids and phenolic compounds (Palmqvist and Hahn-Hägerdal 2000b) and they can cause severe reductions of ethanol yield and ethanol productivity by affecting the performance of the fermenting microorganisms (Klinke et al. 2004). In order to avoid these deleterious effects, a detoxification step, such as evaporation, extraction or treatment with certain enzymes to remove the toxic compounds, is often performed following the pretreatment step (Section 1.2.2.1.1; Palmqvist and Hahn-Hägerdal 2000a). But this detoxification step requires additional equipments, agents and time, therefore increases the complexity and costs of the entire process (Almeida et al. 2007). The *in situ* detoxification ability of yeasts such as *Saccharomyces cerevisiae* makes elimination of the detoxification step possible. For example, *S. cerevisiae* is able to reduce furans such as furfural and 5-hydroxymethylfurfural to the low-toxic furfuryl alcohol and 5-hydroxymethylfurfuryl alcohol, respectively (Liu et al. 2004). Some *S. cerevisiae* strains are also able to metabolize some phenolic compounds present in the pretreated lignocellulosic hydrolysate (Larsson et al. 2000). It is therefore

possible that the detoxification of the lignocellulosic material could take place simultaneously with fermentation by some robust yeast strains. The toxicity of the pretreated lignocellulosic biomass greatly depends on the nature of the lignocellulosic biomass, as well as the pretreatment conditions and the nature of the microorganism (Delgenes et al. 1996). In the present study, two common and abundant agricultural byproducts, corn stover and wheat straw, were pretreated under identical conditions using a hydrothermal pre-treatment method employed by Inbicon A/S (DONG Energy subsidiary, Denmark). The differences in the toxicities of these two biomasses to the xylose fermenting yeast *S. passalidarum* were compared.

In addition to the deleterious effects caused by inhibitors, the un-fermented pentose left in the medium by *S. cerevisiae* also adds to the production cost. In most crop residues, the most abundant pentose is xylose. For example, xylan makes up around 20% in corn stover biomass (Xu et al. 2009) and around 25% in wheat straw biomass (Georgieva et al. 2008). Therefore, efficient conversion of xylose into ethanol is necessary to make the process economically attractive. In our previous study, yeast *Spathaspora passalidarum* showed excellent performance on fermentation of glucose and xylose under anaerobic conditions. This is an important merit of this strain and it is superior to other xylose fermenting yeasts which often require microaerophilic conditions (Section 1.3.2.1.3 and Chapter 2). However, *S. passalidarum* also has a weakness in being sensitive to pretreatment generated inhibitors (Chapter 3). However, provided that the inhibitor tolerance of this yeast can be improved to a suitable level it might become a strong candidate as the best microorganism for the lignocellulosic bioethanol production. Hybrid strains, FS2 and FS22, obtained through mutagenesis of *S. passalidarum* and subsequent protoplast fusion with *S. cerevisiae* ATCC 96581 showed noticeable improved inhibitor tolerance towards the inhibitors present in 75% WSLQ (Chapter 4). The possibility of using these hybrid strains for lignocellulosic bioethanol production was tested in the current study.

An ideal fermentation process involves complete fermentation of the sugars present in pretreated lignocellulosic material, mainly glucose and xylose, without need for a detoxification step. In this study, two fermentation strategies were tested using un-detoxified pretreated wheat straw. The first one used yeast strains alone that having the capacity to utilize both glucose and xylose (i.e. *S. passalidarum* or FS2 or FS22). The second one used a robust yeast, the adapted *S. cerevisiae* ATCC 96581, to detoxify part of the inhibitors simultaneously with fermenting glucose, followed by inoculation of a less robust yeast i.e. *S. passalidarum*, FS2 or FS22 to ferment the residual xylose. The latter strategy was termed “co-fermentation”.

## 5.3 Materials and Methods

### *Yeast cultivations and culture medium*

*Saccharomyces cerevisiae* ATCC 96581, *Saccharomyces cerevisiae* Ethanol Red, *Spathaspora passalidarum* ATCC MYA-4345, and the hybrid strains FS2 and FS22 were used in this study. *S. cerevisiae* Ethanol Red was purchased from Fermentis (Division of S. I. LeSaffre, France) and it is a commercial yeast developed for ethanol industry with claims of high ethanol tolerance, high osmotolerance and a wide range of fermentation temperatures from 30°C to 40°C (Product instruction of Ethanol Red, Fermentis). *S. cerevisiae* ATCC 96581 and *S. passalidarum* ATCC MYA-4345 were from American Type Culture Collection (ATCC). *S. cerevisiae* ATCC 96581 is a heavily flocculating yeast isolated from a sulfite liquor fermentation plant (Linden et al. 1992). *S. passalidarum* is a xylose-fermenting yeast originally associated with wood-boring beetles (Nguyen et al. 2006). The hybrid strains FS2 and FS22 were obtained by fusion of the protoplasts of *S. cerevisiae* ATCC 96581 and a furfural-resistant mutant of *S. passalidarum* (Chapter 4).

The propagation and pre-culture preparation of the strains were according to the procedure described in Chapter 2. Medium used for culturing the yeast strains is the yeast peptone dextrose medium (YPD) containing 10 g/L yeast extract, 20 g/L peptone and 30 g/L glucose.

### ***Pretreated corn stover and wheat straw***

Corn stover or wheat straw with a dry matter (DM) content of 20% was pretreated in the reactor of the pilot plant of Inbicon A/S (DONG Energy subsidiary, Denmark) using a hydrothermal method at 190°C for 12 minutes. After the pretreatment, the slurry was separated into a solid fraction and a liquid fraction. The liquid fraction contains most of the inhibitors generated after pretreatment and was used as the inhibition medium in this study. Table 5.1 lists the main composition of the liquid fraction of pretreated corn stover (CSLQ) and wheat straw (WSLQ).

The solid and liquid fractions of pretreated wheat straw were mixed for separate hydrolysis and fermentation (SHF) by different yeast strains in this study. Dry matter content of this mixture was  $9.4\% \pm 1.1\%$ , as measured by drying the samples for 24 hours at 105°C. The composition of the raw wheat straw material (Table 5.2) was determined by a two-step acid hydrolysis method as described by Xu et al. (2009).

**Table 5.1** Main composition in the liquid fraction of pretreated corn stover (CSLQ) and wheat straw (WSLQ) <sup>a</sup>

Composition Liquid fraction	Furfural (g/L)	HMF (g/L)	Acetic acid (g/L)	Lactic acid (g/L)	Formic acid (g/L)	Total phenols (g/L)	Free glucose (g/L)	Free xylose (g/L)
CSLQ	1.35 ± 0.11	0.20 ± 0.00	4.59 ± 0.14	0.92 ± 0.01	2.54 ± 0.02	4.06 ± 0.37	0.73 ± 0.05	2.03 ± 0.05
WSLQ	0.54 ± 0.01	0.29 ± 0.01	3.39 ± 0.08	0.61 ± 0.01	1.28 ± 0.04	2.86 ± 0.28	1.72 ± 0.03	2.29 ± 0.07

<sup>a</sup>. Data presented are the average of triplicate measurements ± the standard deviations

**Table 5.2** Main composition of raw wheat straw <sup>a</sup>

Composition	Glucan	Xylan	Arabinan	Lignin	Residues	Sum
Content (g/g-DM)	0.39	0.22	0.03	0.20	0.10	0.94

<sup>a</sup>. Data presented are average of duplicate measurements

### ***Batch fermentation in CSLQ and WSLQ***

All batch fermentations were performed with 50 ml working volume in 100 ml anaerobic vials. The inhibition medium contained CSLQ or WSLQ in amounts of 25%, 50%, 75%, and 100%, achieved by diluting CSLQ or WSLQ with addition of Milli-Q water. Extra glucose and/or xylose were added to the media as indicated. pH of the media were adjusted to 5.1 before fermentation and were not controlled during the fermentations. The vials were inoculated with a pre-culture of *S. cerevisiae* ATCC 96581 or *S. cerevisiae* Ethanol Red at OD<sub>620nm</sub> = 0.2, and with *S. passalidarum* or hybrid FS2 or FS22 at OD<sub>620nm</sub> = 0.5, unless otherwise specified. In order to facilitate cell growth at the start stage of fermentation, remaining oxygen in the media and head space of the vials was not flushed out. The vials were incubated at 32°C in an orbital shaker at 120 rpm. Samples were taken under sterile conditions, at certain time intervals.

For the co-fermentation of 100% CSLQ with 30 g/L glucose and 30 g/L xylose, the adapted *S. cerevisiae* ATCC 96581 was first inoculated at OD<sub>620nm</sub> = 0.2, and after 72 hours *S. passalidarum* or FS22 was inoculated at OD<sub>620nm</sub> = 0.5. The vials were incubated at 32°C in an orbital shaker at 120 rpm. Samples were taken under sterile conditions, at certain time intervals.

### ***Adaptation***

Cells of *S. cerevisiae* ATCC 96581 were sequentially transferred to the inhibition media with increasing concentrations of CSLQ (i.e. 75%, 85%, 90% and 100%) containing 30 g/L glucose. Cells were incubated in anaerobic vials at 32°C in an orbital shaker at 120 rpm. At each

concentration of CSLQ, 10% (v/v) of the cell culture was transferred into fresh medium, and incubated for 48 to 96 hours until production of gas was observed in the vials. This transfer was repeated for 3 times. In the last batch of 100% CSLQ, cells were incubated for 96 hours and collected by centrifugation at 4500 rpm for 5 minutes. Cells were washed once with 0.9% NaCl and plated on YPD agar plates. Single colonies were picked and re-streaked on YPD agar plate. A single colony was picked and transferred to YPD liquid medium and grown overnight. The cell culture was inoculated into 100% CSLQ containing 30 g/L glucose. The cells showed a capacity of fermentation in this medium. This colony was kept on YPD agar and named as “adapted *S. cerevisiae* ATCC 96581”.

#### ***Separate hydrolysis and fermentation in un-detoxified pretreated wheat straw***

Fermentation of pretreated wheat straw was carried out as separate hydrolysis and fermentation (SHF) in 50 ml anaerobic vial with 40 ml working volume. Before the inoculation of yeast strains, enzymatic hydrolysis was carried out for 48 hours in order to release glucose and xylose from the pretreated wheat straw. pH of the pretreated material was adjusted to 5.3 before the enzymatic hydrolysis and pH was not adjusted during the enzymatic hydrolysis and the subsequent fermentation. Enzymes NS50013 and NS50010, provided by Novozymes A/S (Denmark), were applied for the enzymatic hydrolysis. NS50013 contains a wide range of cellulose and hemicellulose degrading enzymes and NS50010 contains mainly  $\beta$ -glucosidase which hydrolyzes cellobiose to glucose. NS50013 was added at an enzyme loading of 20 Filter Paper Unit (FPU)/g-cellulose and NS50010 was supplemented at half of the volume of NS50013. One FPU is defined as the amount of enzyme releasing 1  $\mu$ mole of reducing sugar from cellulose filter paper per milliliter per minute. The vials were incubated at 50°C with an orbital rotation speed of 185 rpm during the enzymatic hydrolysis. After 48 hours, the vials were inoculated with yeast strains and incubated at

32°C in an orbital shaker at 185 rpm. For the fermentation by *S. passalidarum* or hybrid FS2 or FS22, preculture was inoculated at  $OD_{620nm} = 0.5$ . For the co-fermentation, the adapted *S. cerevisiae* ATCC 96581 was first inoculated at  $OD_{620nm} = 0.2$ , and after around 24 hours *S. passalidarum* or FS 2 or FS22 was inoculated at  $OD_{620nm} = 0.5$ . Samples were taken under sterile conditions, at certain time intervals.

### **Chemical analysis**

Glucose, xylose, ethanol, lactic acid, formic acid, acetic acid, furfural, HMF, and total phenol content were analyzed as described in Chapter 4.

## **5.4 Results**

### ***Batch fermentation in CSLQ and WSLQ by S. cerevisiae ATCC 96581, S. cerevisiae Ethanol Red and S. passalidarum***

Batch fermentations with three yeast strains *S. cerevisiae* ATCC 96581, *S. cerevisiae* Ethanol Red and *S. passalidarum* were compared in media containing different amounts (25%, 50%, 75% and 100%) of the liquid fraction of pretreated corn stover (CSLQ). *S. passalidarum* could survive only in the medium containing 25% CSLQ giving an ethanol yield from all available glucose at 0.1 g/g (Figure 5.1). This low value is due to the consumption of only 19% of the available glucose in the medium (data not shown). *S. cerevisiae* ATCC 96581 and *S. cerevisiae* Ethanol Red consumed all the glucose in media containing 25% and 50% CSLQ (data not shown). The ethanol yield of *S. cerevisiae* ATCC 96581 in 50% CSLQ was noticeably higher than that of *S. cerevisiae* Ethanol Red (Figure 5.1). In the medium containing 75% CSLQ, *S. cerevisiae* ATCC 96581 finished the fermentation within 72 hours with an ethanol yield at 0.45 g/g glucose, while *S. cerevisiae* Ethanol Red showed neither cell growth nor ethanol production until after 120 hours in the same medium

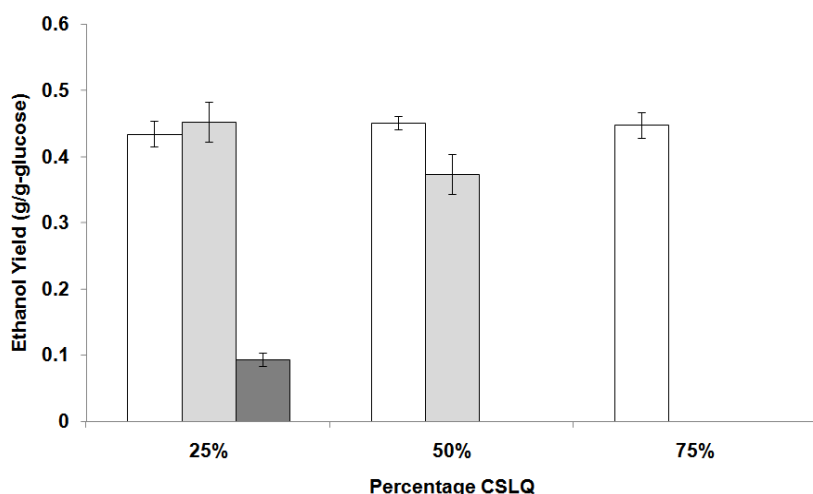


(Figure 5.1). *S. cerevisiae* ATCC 96581 completely converted furfural (1 g/L) and HMF (0.15 g/L) in 75% CSLQ within 24 hours while *S. cerevisiae* Ethanol Red still left 43% furfural after 120 hours (data not shown). *S. cerevisiae* Ethanol Red is tolerant to a range of temperature from 30°C to 40°C and showed excellent fermentation performance at 35°C (Product instruction of Ethanol Red, Fermentis). Therefore, the possibility of a positive effect of temperature on the inhibitor tolerance of this yeast was examined. There was no difference between the fermentation performance of this yeast at 35°C and 32 °C in media containing 50% CSLQ or 75% CSLQ (data not shown). None of the three analysed strains, *S. passalidarum*, *S. cerevisiae* ATCC 96581 and *S. cerevisiae* Ethanol Red, produced ethanol in a medium containing 100% CSLQ. These results demonstrate that *S. cerevisiae* ATCC 96581 is the most tolerant to the inhibitors present in CSLQ and that *S. passalidarum* is much more sensitive than *S. cerevisiae* strains.

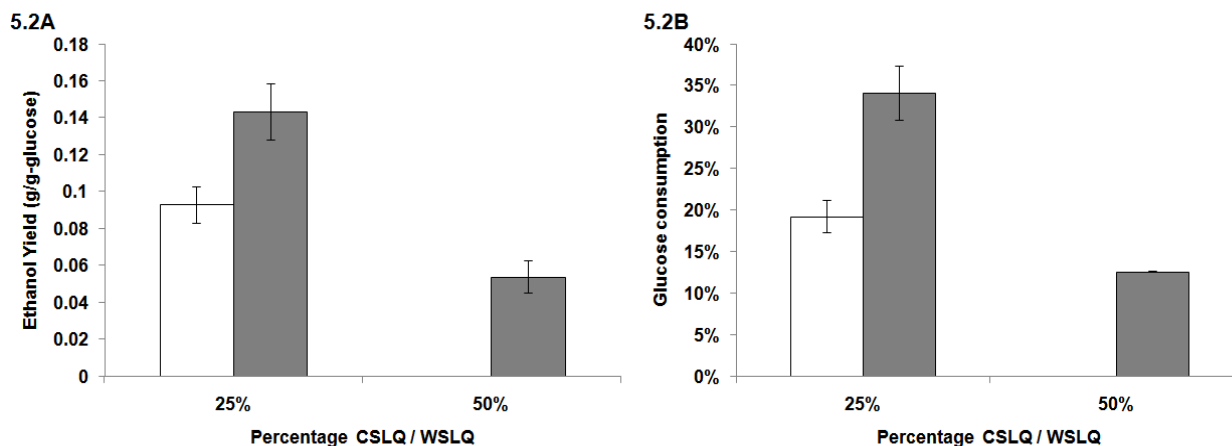
The sensitivity of *S. passalidarum* to the liquid fraction of pretreated corn stover (CSLQ) and wheat straw (WSLQ) were compared. Corn stover and wheat straw with same content of dry matter were pretreated under the identical conditions. The amounts of inhibitors released from wheat straw were however lower than those released from corn stover (Table 5.1), and as a consequence WSLQ inhibited *S. passalidarum* less than CSLQ. 34% glucose was consumed and ethanol was produced at a yield of 0.16 g/g total glucose by *S. passalidarum* in the medium containing 25% WSLQ, while only 19% glucose was consumed and ethanol was produced at a yield of 0.09 g/g total glucose in the medium containing 25% CSLQ (Figure 5.2). *S. passalidarum* can still ferment 12% glucose in the medium containing 50% WSLQ, producing ethanol at a yield of 0.06 g/g total glucose. In comparison, the strain showed neither sugar consumption nor ethanol production in the medium containing 50% CSLQ (Figure 5.2). Comparing the amounts of analyzed inhibitors, 50% WSLQ were comparable with 25% CSLQ (Table 5.1). The furfural concentrations were slightly lower in 50% WSLQ (0.25 g/L) than in 25% CSLQ (0.32 g/L), but the concentration of total phenols was

slightly higher in 50% WSLQ (1.4 g/L) than in 25% CSLQ (1.0 g/L). The fermentation performance of *S. passalidarum* in 50% WSLQ and 25% CSLQ showed that *S. passalidarum* was more inhibited by 50% WSLQ than by 25% CSLQ (Figure 5.2). Thus, the result indicates that unknown phenolic compounds might be more inhibiting than furfural, or that a synergistic inhibition effect was more pronounced in 50% WSLQ than in 25% CSLQ.

With *P. stipitis* fermenting xylose in the synthetic medium it has been found that increased initial cell concentration leads to enhancement of xylose consumption and a higher ethanol yield (Agbogbo et al. 2007). Therefore, a potential effect of varying initial cell concentrations of *S. passalidarum* on inhibitor tolerance was investigated. However, no obvious difference was found using inoculum concentrations at  $OD_{620nm} = 0.1$ ,  $0.2$  and  $0.5$  during the fermentation of media containing 25% or 50% WSLQ. Only a slightly increased glucose consumption was observed during the fermentation of 50% WSLQ with an inoculum concentration of  $OD_{620nm} = 0.5$  (Table 5.3). A linear correlation between  $OD_{620nm}$  and cell number of *S. passalidarum* was determined with  $OD_{620nm} = 1$  corresponding to approximately  $1 \times 10^7$  cells/ml.



**Figure 5.1** Ethanol yields from available glucose (30 g/L) in the media containing different concentrations (25%, 50% and 75%) of CSLQ after 120 hours of anaerobic batch fermentation by *S. cerevisiae* ATCC 96581 (white column), *S. cerevisiae* Ethanol Red (gray column) and *S. passalidarum* (black column). Data presented are average of triplicate experiments and error bars indicate the standard deviations.



**Figure 5.2** Ethanol yield from available glucose (30 g/L) in the medium (**2A**) and glucose consumption (**2B**) after 144 hours of anaerobic batch fermentation by *S. passalidarum* in the medium containing 25% or 50% of CSLQ/WSLQ; CSLQ (light column), WSLQ (dark column). Data presented are average from duplicate experiments and error bars indicate the ranges of measurements.

**Table 5.3** Variation of the inoculum size of *S. passalidarum* during the anaerobic fermentation of 25% and 50% WSLQ <sup>a</sup>

Percentage WSLQ	Inoculum Concentration (OD <sub>620nm</sub> )	Glucose consumption (%)	Ethanol yield (g/g-consumed glucose)	Carbon recovery (%)
25%	0.1	29.0 ± 3.1	0.41 ± 0.01	90.3 ± 4.2
	0.2	30.9 ± 1.1	0.37 ± 0.02	85.2 ± 1.7
	0.5	30.9 ± 2.6	0.40 ± 0.00	91.0 ± 2.2
50%	0.1	12.7 ± 0.8	0.48 ± 0.03	98.1 ± 2.5
	0.2	10.2 ± 2.6	0.45 ± 0.01	92.8 ± 1.0
	0.5	15.3 ± 0.7	0.41 ± 0.01	90.2 ± 0.1

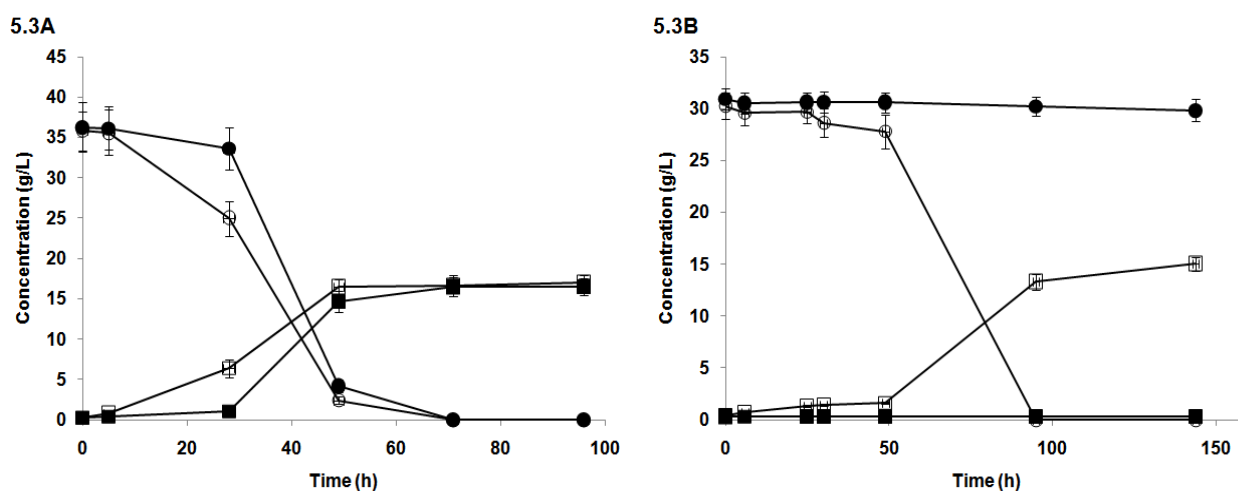
<sup>a</sup> All media contained glucose at 30 g/L. Values are calculated after 72 hours of fermentation. Data presented are average of duplicate experiments ± range of measurements.

### **Batch fermentation in CSLQ by the adapted *S. cerevisiae* ATCC 96581 and its co-fermentation with *S. passalidarum* or FS22**

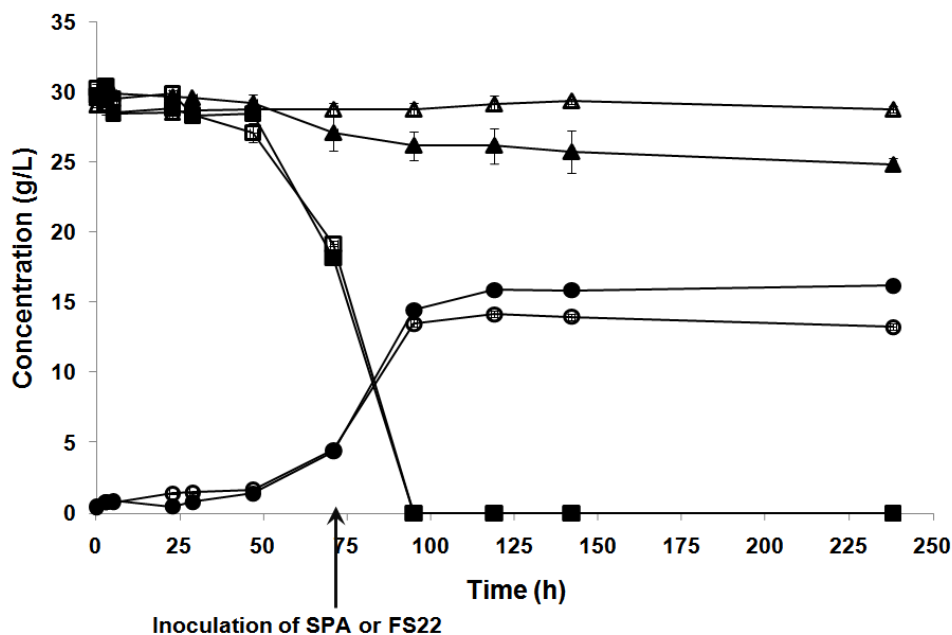
The inhibitor tolerance of the most robust strain, *S. cerevisiae* ATCC 96581, was further improved by sequentially adapting the strain in media containing increasing amounts of CSLQ (from 75% to 100%). The resulting adapted strain exhibited a shorter lag phase than the parental strain in the medium containing 75% CSLQ (Figure 5.3A). In a medium containing 100% CSLQ,

the adapted strain exhibited a lag phase of 49 hours and finished the fermentation within 96 hours with an ethanol yield at 0.48 g/g glucose, while the parental strain was still inhibited after 144 hours (Figure 5.3B). During the fermentation of 100% CSLQ, the adapted strain converted all furfural and HMF, while the parental strain only converted 43% furfural and 30% HMF after 144 hours (data not shown).

Fermentation of 100% CSLQ medium by the adapted *S. cerevisiae* ATCC 96581 followed by inoculation with *S. passalidarum* did not improve the ethanol yield, as no xylose consumption was observed after *S. passalidarum* had been inoculated (Figure 5.4). This result indicates that although all furfural and HMF had been converted by the adapted strain of *S. cerevisiae* ATCC 96581, *S. passalidarum* was still inhibited, probably by other inhibitors such as weak acids or unknown phenolic compounds present in CSLQ. A similar co-fermentation of the adapted *S. cerevisiae* ATCC 96581 with the hybrid FS22 improved the ethanol yield from 0.23 g/g total sugars to 0.27 g/g total sugars, due to the additional ethanol production from xylose by the hybrid strain FS22 (Figure 5.4). The hybrid consumed 16% of 30 g/L xylose in 100% CSLQ medium.



**Figure 5.3** Glucose consumption and ethanol production during the anaerobic fermentation of *S. cerevisiae* ATCC 96581 and the adapted strain in the medium containing 30 g/L glucose and 75% CSLQ (**5.3A**) or 100% CSLQ (**5.3B**); glucose consumed by the parental strain (*filled circle*), ethanol produced by the parental strain (*filled square*), glucose consumed by the adapted strain (*open circle*), ethanol produced by the adapted strain (*open square*). Data presented are average from duplicate experiments and error bars indicate the ranges of measurements.



**Figure 5.4** Co-fermentation of the adapted *S. passalidarum* ATCC 96581 with *S. passalidarum* or hybrid FS22 in 100% CSLQ containing 30 g/L glucose and 30 g/L xylose. *S. passalidarum* and hybrid FS22 were inoculated after 72 hours fermentation by the adapted *S. cerevisiae* ATCC 96581, as indicated in the figure; glucose consumed by FS22 (filled square), glucose consumed by *S. passalidarum* (open square), xylose consumed by FS22 (filled triangle), xylose consumed by *S. passalidarum* (open triangle), ethanol produced by FS22 (filled circle), ethanol produced by *S. passalidarum* (open circle). Data presented are average of duplicate experiments and error bars indicate the ranges of measurements.

### *Separate hydrolysis and fermentation in un-detoxified pretreated wheat straw*

Separate hydrolysis and fermentation (SHF) was carried out for the fermentation of pretreated wheat straw in this study. Raw wheat straw contained around 0.39 g/g-DM of cellulose (glucan) and 0.22 g/g-DM of hemicellulose (mainly xylan) (Table 5.2). After 48 hours of enzymatic hydrolysis at an enzyme load of 20 FPU/g-cellulose, around 30 g/L glucose and 16 g/L xylose were released, corresponding to 70% of cellulose conversion and 66% of hemicellulose conversion.

After enzymatic hydrolysis, two kinds of fermentation strategies were tested. The first one was inoculating alone the yeast *S. passalidarum* or FS2 or FS22 having the capacity for both glucose and xylose utilization. The wild type *S. passalidarum* was almost unable to ferment in the hydrolysate, as only 2.8% glucose was consumed and no ethanol production was detected. The hybrid strain FS2 and FS22 showed much better performance, with glucose conversion at 33% and

42%, respectively (Table 5.4). However, no xylose was consumed by either of these three strains, which could be due to glucose repression of the xylose uptake (Chapter 2). Ethanol yields from consumed glucose were 0.36 g/g and 0.41 g/g using FS2 and FS22, respectively. Furfural (~0.5 g/L) present in the pretreated wheat straw hydrolysate was converted within 72 hours by all the three strains and the furfural reduction rate by FS22 at the first 48 hours was detected to be the highest (Table 5.4). The second strategy was inoculating the adapted *S. cerevisiae* ATCC 96581 first, to detoxify part of the inhibitors such as reducing furfural simultaneously with fermenting glucose. After 24 hours when most furans had been converted, the xylose fermenting yeast strains were then inoculated to ferment xylose. The detoxification by *S. cerevisiae* improved the cell growth of *S. passalidarum* wild type in the hydrolysate, resulting in 8% xylose consumption, however no obvious improvement on ethanol yield was observed. In comparison, the hybrids FS2 and FS22 improved the final ethanol yield by 11% and 26%, respectively, due to the partial conversion of xylose into ethanol by these two strains (Table 5.5).

**Table 5.4** Anaerobic batch fermentation of un-detoxified pretreated wheat straw hydrolysate by *S. passalidarum* and hybrids FS2 and FS22

Strain	Glucose in the hydrolysate (g/L) <sup>a</sup>	Xylose in the hydrolysate (g/L) <sup>a</sup>	Glucose consumption (%) <sup>b</sup>	Final ethanol yield (g/g-consumed sugar) <sup>b</sup>	Average furfural reduction rate (g/l/h) <sup>c</sup>
<i>S. passalidarum</i>	30.4 ± 1.3	16.6 ± 0.4	2.8 ± 0.4	-	0.005 ± 0.001
FS2	27.8 ± 0.9	15.7 ± 0.1	33.1 ± 2.2	0.36 ± 0.02	0.006 ± 0.000
FS22	29.1 ± 0.6	16.5 ± 0.2	41.6 ± 2.9	0.41 ± 0.04	0.007 ± 0.000

<sup>a</sup> Glucose and xylose concentrations were analyzed after 48 hours of enzymatic hydrolysis. Fermentations were started after 48 hours of enzymatic hydrolysis.

<sup>b</sup> Values were calculated after 190 hours of fermentation. No xylose consumption was observed.

<sup>c</sup> Average furfural reduction rates were calculated during the first 48 hours of fermentation.

<sup>~</sup> Not determined.

Data presented are the average of duplicate experiments ± the range of measurements.

**Table 5.5** Co-fermentation of un-detoxified pretreated wheat straw hydrolysate by the adapted *S. cerevisiae* ATCC 96581 with *S. passalidarum* or FS2 or FS22

Co-fermentation strain	Glucose in the hydrolysate (g/L) <sup>a</sup>	Xylose in the hydrolysate (g/L) <sup>a</sup>	Xylose consumption (%) <sup>b</sup>	Final ethanol yield (g/g-total sugars) <sup>b</sup>	Ethanol yield improvement (%) <sup>c</sup>
<i>S. passalidarum</i>	29.5 ± 1.8	17.7 ± 0.7	8.3 ± 4.4	0.27 ± 0.01	0
FS2	32.8 ± 0.3	18.9 ± 0.6	31.3 ± 1.6	0.30 ± 0.00	11.3
FS22	28.0 ± 0.7	16.7 ± 0.1	38.9 ± 0.3	0.34 ± 0.01	26.1

<sup>a</sup> Glucose and xylose concentration were analyzed after 48 hours of enzymatic hydrolysis. The adapted *S. cerevisiae* ATCC 96581 was inoculated after 48 hours of enzymatic hydrolysis.

<sup>b</sup> *S. passalidarum* or FS2 or FS22 was inoculated after 24 hours fermentation by the adapted *S. cerevisiae* ATCC 96581. Xylose consumption and final ethanol yield were calculated after 166 hours of fermentation.

<sup>c</sup> Ethanol yield by the adapted *S. cerevisiae* ATCC 96581 alone ( $Y_{E-AA}$ ) was  $0.27 \pm 0.1$  g/g-total sugars. Ethanol yield improvements were calculated as: Improvement = (Final ethanol yield –  $Y_{E-AA}$ ) /  $Y_{E-AA} \times 100\%$

Data presented are average of duplicate experiments ± range of measurements.

## 5.5 Discussion

*S. cerevisiae* has been recognized as the most robust yeast regarding the tolerance to inhibitors present in pretreated lignocellulosic biomass, as compared with bacteria and other yeasts (Hahn-Hägerdal et al. 2007; van Maris et al. 2006). Different *S. cerevisiae* strains can display different tolerances to inhibitors (Almeida et al. 2009). In the present study, *S. cerevisiae* ATCC 96581 showed higher tolerance to inhibitors present in CSLQ than *S. cerevisiae* Ethanol Red. This higher tolerance could be due to the higher capacity of furfural reduction in *S. cerevisiae* ATCC 96581. Another possible reason for the robustness of *S. cerevisiae* ATCC 96581 could be the flocculation properties of this strain. The mechanism of yeast cells adhesion has been studied thoroughly (Almeida et al. 2007; Mukherjee and Chandra 2004). Different adhesion properties provide yeast cells with the ability to adapt quickly to stressful environments (Verstrepen and Klis 2006). It is conceivable that cells in the middle of the flocs are protected from the adverse environment

(Verstrepen and Klis 2006). This may increase the viability of cells and subsequently enhance the rate of *in situ* detoxification of harmful inhibitors.

The xylose fermenting yeast *S. passalidarum* could only survive in the medium containing low amounts of CSLQ or WSLQ, which is in accordance with the previous discovery of low inhibitor tolerance of this yeast (Chapter 3). The extent of inhibition on *S. passalidarum*, as seen by the inhibition on sugar consumption and ethanol production, was less severe in 25% WSLQ than 25% CSLQ. This was due to the lower amounts of inhibitors in pretreated wheat straw as compared with pretreated corn stover. Although corn stover and wheat straw were pretreated under the same condition, the amounts and composition of inhibitors formed were different. This is due to the different compositions of these two kinds of biomass. Corn stover contains around 30% glucan, 20% xylan, 17% lignin, and 7% ash (Templeton et al. 2009). In comparison, wheat straw contains 39% glucan, 22% xylan, 20% lignin, and 6% ash, as determined in this study.

When the concentrations of analysed inhibitors were comparable, i.e. 50% WSLQ and 25% CSLQ, *S. passalidarum* was more inhibited in the medium containing 50% WSLQ than 25% CSLQ. Slightly lower amount of furfural and slightly higher amount of total phenols were present in 50% WSLQ than in 25% CSLQ, and therefore the results indicate that *S. passalidarum* might be more sensitive to the unknown phenol compounds rather than to furfural, although the latter one has been identified as a key toxin and correlates with the toxicity of the pretreated hydrolysate (Heer and Sauer 2008). The degree of inhibition caused by toxic compounds greatly depends on the nature of the microorganism, and different microorganisms can have different sensitivities to one inhibitor (Delgenes et al. 1996). The inhibition mechanism of different inhibitors has only been clarified for a few. Furans such as furfural and HMF have been found to inhibit key enzymes during cell metabolism (e.g. alcohol dehydrogenase, pyruvate dehydrogenase, and aldehyde dehydrogenase) or to reduce intracellular ATP and NAD(P)H levels (Chapter 3; Modig et al. 2002). Weak acids can



lead to the cytoplasmic acidification and ATP depletion (Russell 1992). Phenolic compounds are suggested to be the most toxic compound but their inhibition mechanisms are the least understood (Palmqvist and Hahn-Hägerdal 2000b). One possible explanation of phenol toxicity could be the disturbance of cell membrane integrity (Heipieper et al. 1994). Apart from the possibility of high sensitivity of *S. passalidarum* to some unknown phenolic compounds, possible synergistic effects with other inhibitors cannot be excluded. HMF, formed by dehydration of hexoses, is an important inhibitor to microorganisms ( Ma and Liu 2010; Almeida et al. 2007), but only small amounts (0.2~0.3 g/L) of HMF were released from wheat straw and corn stover after the hydrothermal pretreatment applied in this study. HMF can be formed in extremely high concentrations, especially under the acidic pretreatment conditions (Klinke et al. 2004), while it can be almost absent under the wet-oxidation conditions (Almeida et al. 2007). This low amount of HMF (0.2~0.3 g/L) may not be the reason for the severe inhibition on *S. passalidarum* but could possibly act synergistically with other inhibitors.

The inhibitor tolerance of wild type *S. cerevisiae* ATCC 96581 was improved by sequentially adapting the strain to increasing concentration of CSLQ. Improvements of inhibition tolerance of yeast strains by rational design have previously been described, but these strains have not been sufficient to carry out optimal fermentations in real lignocellulosic hydrolysate (Heer et al. 2009; Almeida et al. 2008; Larsson et al. 2001). The reason is the complicated nature of microbial inhibition with many inhibitors acting alone or in synergy, requiring numerous genetic alterations to achieve the desired effects (Ma and Liu 2010; Laadan et al. 2008; Liu et al. 2008; Gorsich et al. 2006). Therefore alternative methods such as genome shuffling (e.g. via random mutagenesis and protoplast fusion) with directed selection (Chapter 4), or directed adaptation that mimics the natural evolution for the selection of desired phenotypes (this study) can be applied to improve inhibitor tolerance of different yeast strains. These methods are especially useful for the improvement of

desired phenotypes of industrial strains when the knowledge of the underlying mechanisms is lacking or too complex.

Finally, *S. passalidarum* wild type and two tolerance improved hybrids FS2 and FS22 (Chapter 4) were tried in the un-detoxified pretreated wheat straw, and it is the first time to describe the performance of *S. passalidarum* in the real lignocellulosic material. *S. passalidarum* wild type was found to be almost completely inhibited in the pretreated wheat straw hydrolysate, while the two hybrids showed significantly improved fermentation performance. However, using the hybrids alone for lignocellulosic bioethanol fermentation is still far away from industrial requirements, as around 60% of glucose was left unconsumed and no xylose consumption had occurred during the fermentation of pretreated wheat straw. The failure to utilize xylose could be due to the unfermented glucose repressed the xylose fermentation (Chapter 2). By co-fermentation with the adapted *S. cerevisiae* ATCC 96581, the hybrids FS2 and FS22 noticeably improved the final ethanol yield by converting part of the xylose into ethanol.

As a conclusion, this work showed that the co-fermentation strategy of using a robust yeast strain to detoxify some of the inhibitors and at the same time ferment C6-sugar can facilitate fermentation with a C5-sugar-fermenting yeast and thus improve the total ethanol yield. However, xylose fermenting yeast *S. passalidarum* is very sensitive to the inhibitors present in pretreated biomass. Partial detoxification by *S. cerevisiae* can only partly facilitate the growth of *S. passalidarum*, possibly due to the sensitivity of *S. passalidarum* to other unconverted inhibitors. Better fermentation performance by the hybrids may be because of the increased inhibitor tolerance of these strains to the unconverted inhibitors. In order to utilize these xylose fermenting yeast strains in real lignocellulosic bioethanol production, further investigation to clarify the inhibition mechanism in *S. passalidarum* is required.

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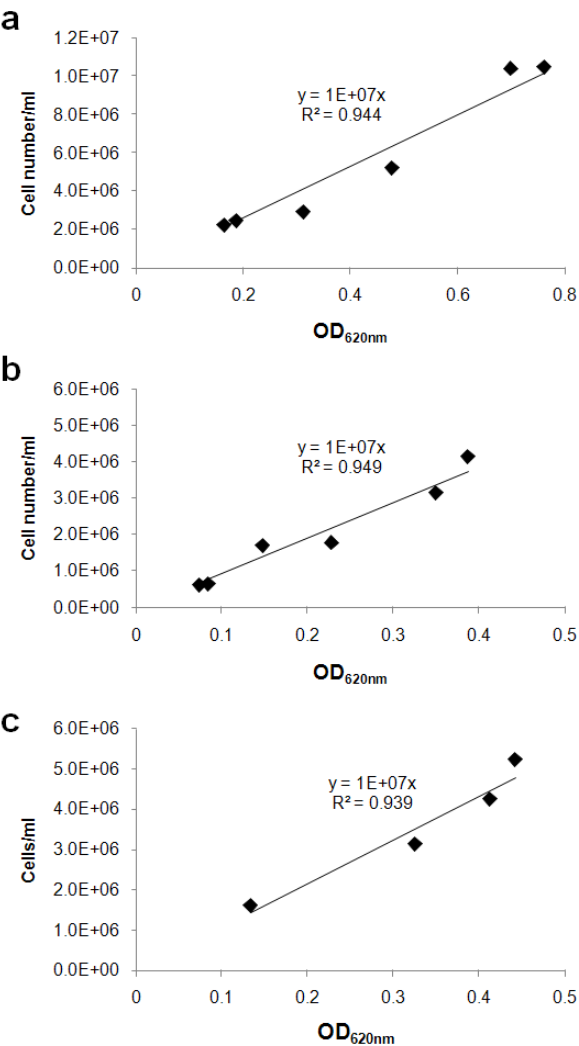
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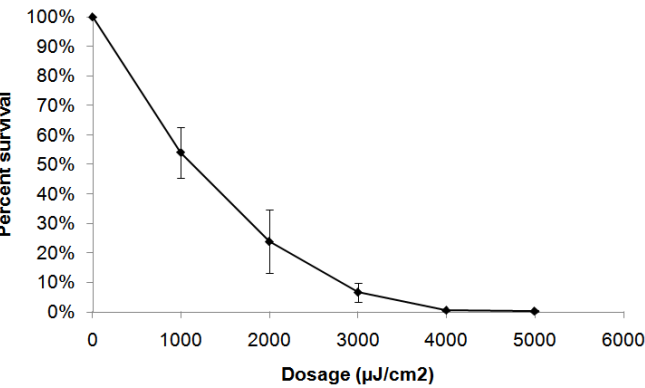
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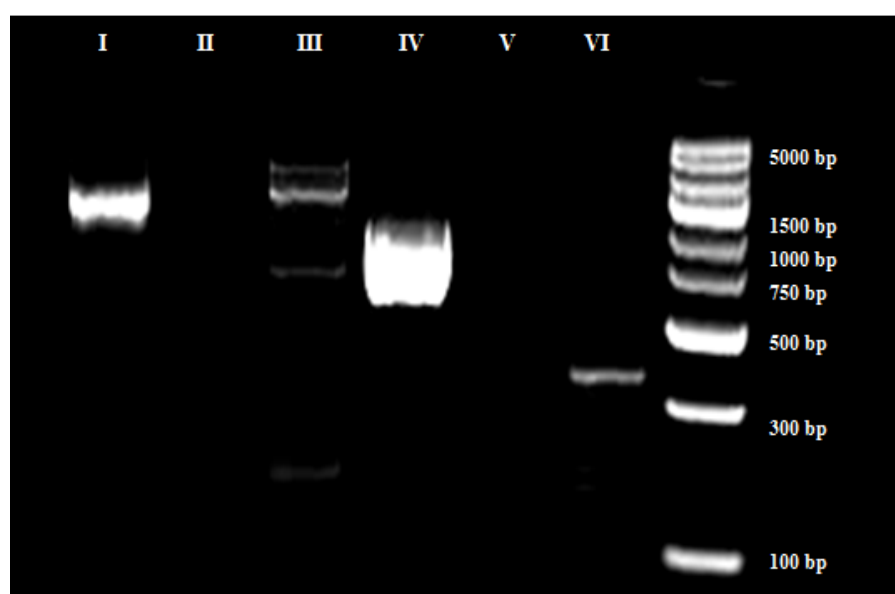
# Appendix



**Figure A1**  $OD_{620nm}$  and cell number relationship of *S. passalidarum* in HDD (a), HDX (b) and YPD (c) medium. Data points are average from duplicate experiments.



**Figure A2** Survival curve of *S. passalidarum* exposed to different dosages of UV light. Data points are average of triplicate experiments and error bars indicate the standard deviations.



**Figure A3:** PCR amplification of ALD5 and SFA1 gene regions in *S. cerevisiae* by using chromosomal DNA templates of *S. cerevisiae* ATCC 96581, *S. passalidarum* M7 and the hybrid strain FS22. **Lane I:** *S. cerevisiae* chromosomal DNA as template using primers of Sac\_ALD5\_fr and Sac\_ALD5\_re; **Lane II:** *S. passalidarum* chromosomal DNA as templates using primers of Sac\_ALD5\_fr and Sac\_ALD5\_re; **Lane III:** FS22 chromosomal DNA as templates using primers of Sac\_ALD5\_fr and Sac\_ALD5\_re; **Lane IV:** *S. cerevisiae* chromosomal DNA as templates using primers of Sac\_SFA1\_fr and Sac\_SFA1\_re; **Lane V:** *S. passalidarum* chromosomal DNA as templates using primers of Sac\_SFA1\_fr and Sac\_SFA1\_re; **Lane VI:** FS22 chromosomal DNA templates using primers of Sac\_SFA1\_fr and Sac\_SFA1\_re; **Lane M:** Gene Ruler™ Express DNA ladder (Fermentas, Denmark)

**Table A1** Sequences of the primers and the amplified regions

Primer name	Sequence (5'→3')	Amplified region	Size of the amplified fragments (bp)
Sac_ALD4_fr Sac_ALD4_re	GTTCAGTAGATCTACGCTCTG CCCACCGAAAGGAACTGC	Ald4p(ALD4) mRNA in <i>S. cerevisiae</i> S288C	1465
Sac_ALD5_fr Sac_ALD5_re	CTGCAGCTCCGAATTCCAG CCTGACTGGCCGAAGCC	Ald5p (ALD5) mRNA in <i>S. cerevisiae</i> S288C	1455
Sac_SFA1_fr Sac_SFA1_re	CTGCTGTTGCGTATGATGCG CAGTGACCAGCTGGAACGG	Sfa1p (SFA1) mRNA in <i>S. cerevisiae</i> S288C <sup>a</sup>	915
Sac_ADH6_fr Sac_ADH6_re	GGTATCGCTATTCAATCACACG CCTTTCGAAGGCTTCATGGAC	Adh6p(ADH6) mRNA in <i>S. cerevisiae</i> S288C	993
Sac_ADH7_fr Sac_ADH7_re	CAGGGCATCGGTATTTCCAAC GGCTGACGCCTTCTTCGC	Adh7p(ADH7) mRNA in <i>S. cerevisiae</i> S288C	985

<sup>a</sup>. Sfa1p is a bifunctional enzyme containing both alcohol dehydrogenase and glutathione-dependent formaldehyde dehydrogenase activities, functions in formaldehyde detoxification and formation of long chain and complex alcohols.



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Xiaoru Hou

Roskilde, Denmark

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## List of Publications

**I:** The results based on Chapter 2 have been published in the following research article:

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

### **Anaerobic xylose fermentation by *Spathaspora passalidarum***

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

### **Improved inhibitor tolerance in xylose-fermenting yeast *Spathaspora passalidarum* by mutagenesis and protoplast fusion**

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